IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hermona Soreq et al.	Atty Docket:	2391-00101
Serial No.:	09/980,263	Art Unit:	1647
Date Filed:	March 21, 2002	Examiner:	Wegert, Sandra L.
Invention:	Diagnostic Uses of Antibodies Against Acetylcholinesterase or C-terminal Peptides Thereof		

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CERTIFICATE OF MAILING

I hereby certify that this document, along with any other papers referred to as being attached or enclosed, is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on November 5, 2004.

Amy E. Rinaldo

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF SOREQ, PH.D. IN SUPPORT OF APPLICANT'S RESPONSE OF NOVEMBER 5, 2004 [37 C.F.R. § 1.132]

Dear Sir:

In support of the Response filed on November 5, 2004 submitted in reply to the Office Action of August 5, 2004 ("Office Action") for the above-referenced matter, I hereby declare as follows:

1. My name is Hermona Soreq, Ph.D. I am a Professor of Molecular Biology at The Hebrew University of Jerusalem, Israel, in the Department of Biological Chemistry. I am currently the Vice-Dean for Research and Development of the Faculty of Natural Sciences, and the Head of the Eric Roland Center for Neurodegenerative Diseases,

both at The Hebrew University. I have published extensively in the field of neurochemistry and molecular neurobiology research, and more particularly my research has focused on acetylcholinesterase biology, with over 100 research publications to date, besides numerous reviews and book chapters. I am also an inventor or co-inventor of a substantial number of patents and patent applications involving, *inter alia*, various AChE transgenic mice, genetically engineered human cholinesterases, AChE-derived peptides, and I am a co-inventor of the invention claimed in the current application. My further credentials are set forth in my Curriculum Vitae, which is attached hereto as **Exhibit A**.

2. I have read the Office Action of August 5, 2004. This declaration is submitted so as to clarify the record concerning stress, stress disorders, and more specifically, central nervous system stress. In particular, I intend to provide clear and convincing evidence that the term "stress" is well known to the man skilled in the art, that the stress swim test performed in the present invention is a reliable and widely used test to measure "stress" in animals and that, therefore, that it is reasonable to claim, based on the support provided by the present invention, that the antibody of the invention is readily usable for diagnosing central nervous system stress.

3. For the past 10 years (at least) my work has focused on uncovering the molecular mechanisms that underlie *stress responses* in humans. These responses are necessary to sustain normal cognitive and behavioral functions, and facilitate responses to altered internal and environmental conditions. Multiple neurological and behavioral characteristics are modified under *stress* and affect the health and well-being of humans; research approaches to these responses need to combine state-of-the-art

technologies in molecular neuroscience with cognitive and behavioral observations and genetic engineering techniques. Using such an interdisciplinary approach, my research is beginning to unravel the molecular complexities of mammalian behavioral responses to stress and identify genetic determinants of human stress responses.

4. So, for the man skilled in the art, what is the definition of *stress*? Stress is understood as a physiological (and psychological, when referring specifically to humans) state, which is triggered in response to altered internal and environmental conditions such as, for example, exposure to chemical stressors (e.g. poisonous organophosphate insecticides), immunological agents (e.g. bacterial lipopolysaccharide) or experiencing a terror attack. This "physiological state" is the organism's response to said triggering factors, and it might reveal itself in the form of transiently enhanced release of acetylcholine (ACh), erratic behavior following circadian light/dark shift, progressive muscle fatigue and degeneration of neuromuscular junctions, progressive failure of learning and memory, and development of neuropathologies [Soreq, H. *et al.* (2004) Acetylcholinesterase as a window onto stress responses. In: Seckler, Kalin and Reul (eds.) Handbook of Stress and the Brain. Part 1: The Neurobiology of Stress. Elsevier, Amsterdam. **Exhibit B**]. In sum, there are a series of manifestations that together are described by a word "stress". These manifestations, or responses, are primarily (and ultimately) regulated by the central nervous system (CNS) and thus, are also denominated CNS stress.

5. One of the most significant achievements of my research, in the past six years, has been the demonstration that, under stress conditions (i.e., altered internal or environmental conditions), there is a switch in the ratio between AChE-S (the synaptic

form of AChE) and AChE-R (the readthrough form of AChE, the C-terminal cleavable peptide of which protein is also referred to in the instant application as "AChE Readthrough Peptide", or"ARP"), demonstrated by the increased levels of AChE-R mRNA and/or protein in various tissues. This has been described, *inter alia*, in Kaufer *et al.* [Kaufer *et al.* (1998) *Nature* **393**:373-377; **Exhibit C**].

6. As mentioned before, in response to the Office Action of January 5, 2004, we have demonstrated that this switch is a reliable indicative of CNS stress irrespective of what triggered this condition. This is based on several publications from my laboratory, for example Kaufer *et al.* (1998), Shohami *et al.* (2000), Cohen *et al.* (2002), Tomkins *et al.* (2002), Cohen *et al.* (2003), and Nijholt *et al.* (2004), as will be detailed below.

7. Kaufer *et al.* [Kaufer, D. *et al.* (1998) *id ibid.*] demonstrate how chemical stress (exposure to cholinesterase inhibitors like disiopropylfluorophosphate or pyridostigmine) induce increased AChE-R expression (see Figures 4 and 5).

8. Shohami *et al.* [Shohami, E. *et al.* (2000) *J. Mol. Med.* **78**:228-236; **Exhibit D**] describe how in response to a physical insult, in this case close head injury (CHI), mice exhibit accumulation of AChE-R mRNA (Fig. 1a-d). Moreover, this accumulation was inhibited by an antisense oligonucleotide specific to AChE-R (Fig. 1e-j).

9. Cohen *et al.* [Cohen, O. *et al.* (2002) *Mol. Psychiatry* **7**:874-885; **Exhibit E**] showed how hAChE-S transgenic mice, which constitutively overexpress the endogenous AChE-R, had impaired adjustment to day-to-night switches (circadian rhythm insult) (Fig. 3a-c).

10. Tomkins *et al.* [Tomkins, O. *et al.* (2002) *Cell. Mol. Neurobiol.* **21**(6): 675-691; **Exhibit F**] showed elevated levels of AChE-R protein in the cerebrospinal fluid of humans suffering from a neurological disease and that presented disruption of the blood-brain barrier (see Fig. 4B).

11. Cohen *et al.* [Cohen, O. *et al.* (2003) *J. Mol. Neurosci.* **21**: 195-208; **Exhibit G**] report a study in humans that were administered endotoxin, wherein there is an increase in plasma levels of a AChE-R cleavage product, ARP, which was associated with impairment of declarative memory (see Fig. 3).

12. Nijholt *et al.* [Nijholt, I. *et al.* (2004) *Mol. Psychiatry* **9**:174-183; **Exhibit H**] demonstrate a correlation between enhanced fear memory (an evolutionary advantageous memory of events that are potentially threatening to the organism) and AChE-R expression (see Fig. 2a).

13. Thus, based on our findings, we have established a paradigm, wherein increased levels of AChE-R, either mRNA or protein (or both), i.e. the switch in the AChE-S/AChE-R ratio, may be used as a marker for stress.

14. Numerous scientists, in the fields of Molecular Neurobiology, Neurophysiology, Toxicology, Psychology, Pharmacology and Psychiatry study the phenomena of *stress*. The "confined swim protocol" or "swim stress test" has been used and cited as a classical protocol for evaluating *stress responses* in mice, see for example Duncan *et al.* [Duncan, G. E. *et al.* (1998) *J. Pharmacol.* **285**: 579-587; **Exhibit I**]. Thus, animals that underwent this protocol are generally considered as good model systems for "stressed animals", and the study of any physiological response recorded in these animals may be interpreted as a "stress response".

15. Thus, because of its credibility in the scientific community, we applied the confined swim protocol as a "stress test", in order to evaluate whether the levels of ARP (the AChE-R protein isoforms) were elevated under such conditions. And indeed, as shown in Fig. 3 (lane 4) ARP was detected in said stressed mice, and not in mice that were not subjected to said test. The detection of the ARP was performed using the anti-ARP antibody claimed in the instant invention.

16. In conclusion, considering that (i) the confined swim protocol is a well accepted protocol for inducing stress (or CNS stress) in mice, (ii) elevation in AChE-R levels is a molecular marker of CNS stress, and (iii) the antibody of the instant invention specifically recognizes the ARP protein, it is reasonable to claim that the antibody of the present invention is for diagnosing CNS stress.

17. I hereby declare that all statements made herein are of my own knowledge and that all statements made on information and belief are true; and further that these statements are being made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Hermona Soreq, Ph.D.

Dated: November 4, 2004

Exhibits:

A - *Curriculum Vitae* of Hermona Soreq, Ph.D.

B – Soreq, H. *et al.* (2004) Acetylcholinesterase as a window onto stress responses. In: Seckler, Kalin and Reul (eds.) Handbook of Stress and the Brain. Part 1: The Neurobiology of Stress. Elsevier, Amsterdam.

C – Kaufer *et al.* (1998)

D - Shohami *et al.* (2000)

E - Cohen *et al.* (2002)

F - Tomkins *et al.* (2002)

G - Cohen *et al.* (2003)

H - Nijholt *et al.* (2004)

I – Duncan *et al.* (1998)

EXHIBIT A

11/5/2004

HERMONA SOREQ, PHD
PROFESSOR OF MOLECULAR BIOLOGY
THE ALEXANDER SILBERMAN INSTITUTE FOR LIFE SCIENCES
THE HEBREW UNIVERSITY
GIVAT RAM, JERUSALEM 91904 JERUSALEM
TEL. 972-2-658 5109 FAX. 972-2-652 0258 E-MAIL: SOREQ@CC.HUJI.AC.IL
HOME ADDRESS 14 HAMAAYAN ST., EIN KEREM, JERUSALEM 95903 TEL: 972-2-641 4852
FAX: 972-2-642 9333; CELL PHONE: 972-54-337 866

Ph.D. The Weizmann Institute of Science (1976)
Thesis on: "Structure and Functions of Regions in mRNA as Probed by Purified Polynucleotide Phosphorylase".

MAJOR RESEARCH INTERESTS

Stress Responses, Antisense Technology, Acetylcholinesterase Biology, Molecular Neurobiology, Messenger RNA Studies

See Departmental website at http://sites.huji.ac.il/biolchem/soreq.html

PROFESSIONAL POSITIONS

2002 -	Vice Dean for R & D, Faculty of Natural Sciences, The Hebrew University
2000 -	Head, The Eric Roland Center for Neurodegenerative Diseases, The Hebrew University
1995 - 1999	Head, the Alexander Silberman Institute of Life Sciences, The Hebrew University
1992 -1995	Head, Dept. of Biological Chemistry, The Hebrew University
1989 - On	Professor of Molecular Biology, Department of Biological Chemistry, The Hebrew University
1986 - 1988	Associate Professor of Molecular Biology, Department of Biological Chemistry, The Hebrew University of Jerusalem.
1983 - 1986	Associate Professor, Neurobiology Department, Weizmann Institute
1979 - 1983	Senior Scientist, Neurobiology Department, Weizmann Institute
1977 - 1979	Fogarty Fellow, Department of Molecular Cell Biology, The Rockefeller University.
1976 - 1977	Scientist, Biochemistry Department, Weizmann Institute
1969 - 1971	Research Assistant, Biochemistry Department, Weizmann Institute, Rehovot.
1967 - 1969	Research and Teaching Assistant, Department of Biochemistry, Tel Aviv University.

AWARDS AND FELLOWSHIPS

2001	Honorary Professorship, The Maimonides University, Buenos Aires
2000	Research Prize by the Israeli Minister of Health
1999	Kay Prize for Innovative research, The Hebrew University
1996	Doctor of Philosophy honoris causa in Chemistry, University of Stockholm, Sweden.
1995	Visiting Professor, College de France, Paris.
1992	U.S. Army Science Award of excellence, Miami.
1990-	The Charlotte Slesinger Chair on Cancer Studies, The Hebrew University.
1986 - 1989	Berman Fellowship, The Hebrew University.
1985	Chancellor's Distinguished Lectureship, The University of California, Berkeley.
1982	Honorary Guest Lectureship, The European Society for Neurochemistry, Katania.
1980 - 1983	Charles Revson Career Development Chair, The Weizmann Institute.
1977	(1) B. de Rothschild Research Award. (2) H. Weizmann Postdoctoral Fellowship (declined). (3) Fogarty International Research Fellowship.
1974	Feinberg Graduate School Award, The Weizmann Institute.
1968	Shenkar Award, Tel Aviv University.

INTERNATIONAL PLENARY LECTURES (Past 5 years)

2004	British Embassy Conference on DNA (February) 8th International Montreal/Springfield Symposium on Advances in Alzheimer Therapy, Montreal, Canada (April) International Society for Neurochemistry 1st Special Neurochemistry Conference Avignon, France (May) College de France Seminar, Paris, France (July) Fourth Forum of European Neuroscience, Lisbon, Portugal (July)
2003	Research Advisory Committee on Gulf War Veterans Illnesses of the Dept. of Veteran's Affairs Washington DC, USA (February)
2002	International Society of Developmental Neuroscience, Sidney, Australia (February) Alzheimer's Disease, Geneva, Switzerland (April) US Ministry of Defence (DARPA), Lexington, KY (April) American Society of Neurochemistry, Miami Beach, Florida (June) Genomics GIF meeting, Heidelberg (July).

	VII International Cholinesterase Meeting, Pucon, Chile (November)
2001	Mayo Clinic (January)
	Schering, Berlin (March)
	The Fifth International Conference on the Progress of Alzheimer's and Parkinson's Disease, Kyoto, Japan (April)
	The Maimonides University, Buenos Aires, Argentina (May)
	The Gordon Research Conference on Stress induced gene Expansion, Conneticut (July)
	University of Buenos Aires, Argentina (August)
	Ageing and Dementia, Graz (September)
	The Society of Controlled Release, Paris, France (July)
	The Karolinska Institute, Stockholm, Sweden (September)
	The British Society for Cell-Matrix Associations, Newcastle (September)
	The German Society for Biochemistry and Molecular Biology, Munich, Germany (October)
	Medizinsche Gesellschaft Lecture, Erlangen, Germany (October)
	The University of Heidelberg, Germany (December)
	The Winter Series on Aging, Galveston, Texas (March).
	CARES visiting lectureship, Chicago, Illinois (April).
	Japanese Society of Neurology, Tokyo (May).
	The US Society of Neuroscience, Miami, Florida (October).
	The Herman Niemeyer Plenary Lecture, Chilean Society of Biology, Pucon, Chile (November).
1998	The Sixth International Congress on Psychiatric Genetics, Bonn, Germany.
	The Fourth Congress of the Asian Physiological Societies, Brisbane, Australia.

INTERNATIONAL SOCIETIES AND COMMITTEES

2004	Member of CFI Multidisciplinary Assessment Committee MAC (Canada)
2003-	Director, Oligonucleotide Therapeutics Society (OTS)
2000-2005	Council Member, The International Society for Developmental Neuroscience
1999-2001	President, The Israel Society for Biochemistry and Molecular Biology
1999-2002	Member, Federation of European Neuroscience (FENS) Course Committee
1996-2000	Member, EMBO Long term fellowships committee
1996	Observer, European Community Committee on Biotechnology
1993-On	Steering Committee, European Neurobiology Network (ENN).

Council Member: European Society of Neurochemistry (ESN,1992-1996), International Brain-Research Organization (IBRO, 1994-1996), International Society of Neurochemistry (ISN, 2001-2004), International Society of Developmental Neuroscience (ISDN, 2002-2005).

Member: European Molecular Biology Organization (EMBO), Human Genome Organization (HUGO), Federations of American and Israeli Societies for Experimental Biology (FASEB, FISEB), American Societies for Neuroscience & for Pharmaceutical & Experimental therapeutics, Society of Controlled Release.

NATIONAL COMMITTEES

2004-	Member, Israeli National Council for Research and Development
2003-	MAGNET Committee, Ministry of Commerce
2001-	Member, Israel Interdisciplinary Center for Neuronal Computation (ICNC)
2001-	Bioethics Committee, Israel Academy of Sciences and Humanities
2000-2002	Head, Infrastructure Advisory Committee to the Minister of Science
1998-2002	FIRST Committee for interdisciplinary research, the Israel Academy of Sciences and Humanities.
1997	Scientific Visiting Committee, The Ben Gurion University Department of Biology
1995-	Consulting Committees to the Chief Scientist at the Ministry of Health
1995-1999	Biotechnology Committee, assigned by the Minister of Science
1994-1999	Human Genome Committee, assigned by the Israel Academy of Sciences and Humanities.

BOARDS OF DIRECTORS

2003-	Oligonucleotide Therapeutics Society, Inc.
2002-	FIRST fund, the Israel Academy of Sciences and Humanities
1997-2002	The Landau Fund, Mifal HaPayis
1998-	Machteshim Agan (MA Industries)

HEBREW UNIVERSITY COMMITTEES

2002-	Head, R&D Committee, The Faculty of Science.
2002–	Senate Representative in the Board of Governors.
2002–	Board of Directors
2002-	Co-Chair, International Work Group on Stress Responses, The Institute for Advanced Studies (Together with D. Engleberg).
2001-	Genomics Committee
1995-1999	Head, The Life Sciences Institute Committees (R&D, Organization)
1995-2000	Senate Representative in the Board of Governors
1994-1995	Head, Committee for Life Sciences recruitments

1990-1991	Ph.D. Studies Committee Infrastructure Committee

ISRAELI UNIVERSITY COMMITTEES

2001-	Visiting Committee, Ben-Gurion University's Biology Dept. (with I. Chet)
2002-	Ramot Academic Committee for Biomedical Research, Tel Aviv University

INTERNATIONAL CONFERENCE COMMITTEES

Head, Program Committees:

2002	Co-Chair, Joint Meeting on Molecular Neuroscience, Heidelberg Univeristy-Hebrew University
2002	Joint FASEB – IUBMB Conference, Istanbul
1999	Co-Chair, Program Committee, IBRO Conference, Jerusalem.
1998	New Jersey-Israel Conference on Biotechnology, New Jersey.
1994	Tenth Biannual ESN Conference, Jerusalem.
1993	Bath Sheva de Rothschild workshop on Genome Diversity, Kfar Saba.
1991	Eighth Biannual meeting, European Society of Developmental Biology, Jerusalem.
1991	Fourth Biannual meeting, European Oocyte club, Eilat.

Member of Program Committees:

2003	6th International Conference on Alzheimer's and Parkinson's Disease
2002	7th International Symposium on Advances in Alzheimer Therapy, Geneva.
2002	11th International symposium on Cholinergic Mechanisms, St. Maurtiz.
2000	6th International Symposium on Advances in Alzheimer Therapy, Stockholm.
1998	Biannual ESN Conference, St. Petersburg.
1997	International ISN/ASN meeting, Boston.
1997	International Alzheimer and Parkinson's Disease Meeting, Eilat.
1996	Israeli Biotechnology toward the year 2000, Rehovot.
1991	International Society of Neurochemistry (ISN), Montpellier.

EDITORIAL WORK

1991 - 1997	Deputy Chief Editor for Minireviews, Journal of Neurochemistry
1991 - 1997	Associate Editor, Molecular and Cellular Neurobiology
1991	A special issue on cholinesterases, Molecular and Cellular Neurobiology
1984	Molecular Biology Approach to the Neurosciences. IBRO Handbook Series, methods in the neurosciences. Vol. 7. John Wiley and Sons (London, New York).

Membership in Editorial Boards

2003-	Neurodegenerative Diseases
2001-	NeuroMolecular Medicine
2000-2003	Neurobiology of Disease
2000-	Israel Medical Association Journal
1999-	European Journal of Biochemistry, Journal of Applied Toxicology
1997-	International Journal of Molecular Medicine
1995 -1998	Science Spectra
1994 -	CNS Drug Reviews
1993 -	Antisense Research and Drug Development
1986	Journal of Molecular Neuroscience

TEACHING ASSIGNMENTS

Hebrew University:

2002	ETGAR (Honors) students, 1st year.
1999 – on	Selected Topics in Life Sciences Research, 1st year Biology undergraduates.
1994 - on	Molecular Processes in the brain, 3rd year and Research students in Biology.
1992 - on	Molecular Biochemistry, a laboratory course for 3rd year Biology undergraduate students.
1991 - 1995	Recombinant DNA in Biological Research, a laboratory course for honours undergraduate students.
1987 - on	1. Introduction to Molecular Biology Course, 2nd year Biology and Biochemistry students. 2. Intensive Laboratory Workshop on Eukaryotic Gene Expression: From transgene to organism; post-graduate students

International:

2001, 2004, 2005	Teacher, ISN summer course, Buenos Aires, Avignon, Vienna
1998	Co-organizer, EMBO practical course on Molecular Neurophysiology, Jerusalem.
1984	Organizer, EMBO practical course on Molecular Neurobiology, Rehovot.

STUDENTS AND POST-DOCTORAL FELLOWS

A. **M.Sc. Students**

1. **Daniel Eliyahu**, 1980-1982.
   Altered Ontogenesis of Specific proteins in agranullar cerebellar cortex.
2. **Ruti Parvari**, 1980-1983.
   Biosynthesis of acetylcholinesterase in rat brain and embryonic *Torpedo* organ as studied by the expression of its scarce mRNA species in microinjected *Xenopus* oocytes. (Cosupervisor: I. Silman).
   Feinberg Graduate School Award, 1982. Senior Lecturer in Genetics, Ben-Gurion University.
3. **Anat Safran**, 1981-1983.
   Variations in translatable mRNAs during development of the rat cerebellum.
   Feinberg Graduate School Award, 1983.
4. **Margit Burmeister**, 1982-1984.
   Studies on the biosynthesis of epidermal growth factor in microinjected *Xenopus* oocytes. (Cosupervisor: J. Schlesinger) *Minerva Fellowship.* Professor of Genetics, Ann Arbor, MI.
5. **Adi Avni**, 1983-1984.
   Isolation and partial characterization of a human acetylcholinesterase gene identified by homology to the *Drosophila* gene.
   Feinberg Graduate School Special Award, 1984. Senior Lecturer in Biology, Tel Aviv University
6. **Ronit Zamir**, 1986-1988.
   Chromosomal mapping of human cholinesterase genes.
7. **Shlomo Seidman**, 1987-1989.
   Expression and tissue-specific processing of cloned human butyrylcholinesterase in mRNA injected *Xenopus laevis* oocytes.
8. **Efrat Lev-Lehman**, 1990 - 1991.
   Localization of cholinesterase mRNA transcripts in the mammalian brain.
9. **Nilli Galyam**, 1997-1999.
   Neuronal and hematopoietic consequences of antisense acetylcholinesterase suppression. Pollack Award, 1998. *Wolf Award, 1999.*
10. **Nadav Livny**, 1998-1999.
    Genetic and epigenetic contributions toward anticholinesterase insults.
11. **Nelly Gluzman**, 1997-2000.
    Site-directed mutagenesis approach to drug interactions of human cholinesterases.
12. **Danijel AlBajari**, 1997 – 2001.
    Structure-function relationships between acetylcholinesterase and presenilins.
    Boehringer-Ingleheim fellowship, 1998-2000.
13. **Alastaire Grant**, (BSc University College, London), 1999 – 2001.
    Functional polymophisms in the ACHE locus.*UK Friends of HUJ fellowship, 1999-2001.*
    Ph.D. student, University College, London.
14. **Boris Bryk**, 2002-2004.
    Inherited and acquired interactions between ACHE and PON1 polymorphisms modulate plasma acetylcholinesterase and paraoxonase activities.
15. **Deborah Toiber**, 2002-.
    Novel human and murine Acetlycholinesterase variants.
16. **Adi Gefen** (co-superviser: Raz Yirmia, Dept. of Psychology, The Hebrew University). 2003-
    Cholinergic links with cytokine gene expression.
17. Keren Ailon, (BA in Psychology, Haifa University) 2003-
    Stress-associated involvement of the ACHE/PON1 locus.
18. **Shani Ben-Arie**, 2003-
    Stimulus-inducible modulations in neuronal alternative splicing patterns.
19. **Amit Berson**, 2003-
    Acetylcholinesterase gene expression modifications in neurodegenerative diseases.

B. **Ph.D. Students (and thesis topics)**

1. **Averell Gnatt**, 1985-1990.
   Structure-Function relationships in human cholinesterase genes and their protein products.
   Landau Award, 1990 Assistant Professor in Pharmacology, University of Maryland.
2. **Revital Ben-Aziz Aloya**, 1989 - 1993.
   Post-transcriptional regulation of the human acetylcholinesterase gene. Landau Award, 1991.
3. **Gal Ehrlich**, 1989 - 1993.
   Congenital and acquired modulations in the human cholinesterase genes in tumor and healthy tissues. *Golda Meir Award*, 1989, *Pollack Award, 1990.*
4. **Shlomo Seidman**, 1990-1994.

A morphogenic Role for acetylcholinesterase: Heterologous Expression Studies in microinjected embryos of Xenopus laevis. *Magna Cum Laude. Landau Award, 1995.*

5. **Yael Loewenstein-Lichtenstein**, 1990 - 1996.
Molecular dissection of active domains in human cholinesterases.
Pollack Award, 1991, Landau Award, 1994, Human Frontiers Post-doctoral Fellowship, 1996- 1998.

6. **Rachel Beeri-Leibson**, 1991-1997.
Human acetylcholinesterase expression in transgenic mice: An approach to the molecular control of cholinergic responses. *European Neurobiology Network Award, 1995, Lady Davies Post-doctoral Fellowship, 1997.*

7. **Efrat Lev-Lehman**, 1992 - 1997.
Developmental role(s) of acetylcholinesterase revealed by multileveled modulations of ACHE gene expression.
Golda Meir Award, 1990. B. de Rothschild Post-doctoral Fellowship, 1997-1999.

8. **Mirta Grifman**, 1993 – 1998.
Modulation of cholinergic signalling by Antisense Technology.
ISN Travel Fellowship, 1995. Mexican HUJ Friends Fellowship, 1996.

9. **Meira Sternfeld**, 1992 –1999.
Structural and catalytic functions of alternative human cholinesterase variants in native and transgenic systems.
Pollack Award, 1993. Lady Davis Post-doctoral Fellowship, 1999. Lecturer, Haifa University.

10. **Daniela Kaufer**, 1994 –1999.
Molecular mechanisms underlying cholinergic stress responses in mammals.
Pollack Award, 1996. ISN Travel Fellowship, 1997. EMBO Post-doctoral Fellowship, 1999 (declined).
Human Frontiers Post-doctoral Fellowship, 1999. LSRF Post-Doctoral Fellowship, 2002.
Assistant Professor, UC Berkley, 2004.

11. **Michael Shapira**, 1994 – 2000.
Neurogenetics approach to the transcriptional control of acetylcholinesterase production.
Pollack Award, 1995. *Maria-Ascoli Award, 1999. Deans' Post-doctoral Fellowship, 2001. LSRF Post-Doctoral Fellowship, 2004.* Post-Doctoral Fellow, Stanford University.

12. **Eran Meshorer**, M.Sc. (HUJ), 1999-2003.
Delayed molecular consequences of nervous system stress responses: from DNA microarrays to altered neurotransmission pathways. *ISN Travel Award, 2001, 2003. Lichtenstein Award, 2001, ICNC*
PhD fellowship, 2002. Post-Doctoral Fellow, NIH.

13. **Marjorie Pick**, M.Sc. (University of Melbourne), 2000-2004
Hematopoietic functions of acetylcholinesterase-derived C-terminal peptides. (co-supervisors: A. Eldor and E. Naparstec, TAU).

14. **Osnat Cohen**, DVM (HUJ), 1997-
Dissection of Acetylcholinesterase Contributions toward the Cholinergic Control of Mammalian Behavior (co-supervisor: R. Yirmiya, Psychology). *ASPECT Fellowship and Best Paper Award, 2000.*

15. **Noa Farchi**, 1998-2004
The roles of AChE splice variants in neuronal and muscle function: transgenic engineering approach. (co-supervisor: B. Hochner, Neurobiology). *Dean's Award, 1999, 2000, Pollack Award, 1999.*

16. **Ella Sklan**, M.Sc. (Ben Gurion University), 1999-2004
Acetylcholinesterase/paraoxonase genotype and expression predict anxiety scores in Health, Risk Factors, Exercise Training, and Genetics study.

17. **Inbal Mor**, M.Sc. (HUJ), 1998-
Morphogenic functions of acetylcholinesterase variants in terminal differentiation.
Dean's Award, 2000. Pollack Award, 2001.

18. **Irit Shapira**, B.Sc. (in psychology, HUJ), 2000-
Molecular mediators of cognitive responses to psychological and physiological stress. (co-supervisor: R. Yirmiya, Psychology).

19. **Tama Evron**, B.Sc. (HUJ) 1999-
Acetylcholinesterase-associated modulations of immune responses.

20. **Liat Ben Moyal**, (BSc Ben Gurion University), 2001-
Acetylcholinesterase/Paraoxonase interactions increase the risk of insecticide-induced Parkinson's disease.
Hazelkorn fellowship, 2001-2002.

21. **Erez Podoly**, M.Sc. (HUJ), 2002-
Structural implications of AChE's protein-protein interactions
Co-Supervisor: Oded Livneh

C. **M.D. Ph.D. Students (and thesis topics)**

1. **Patrick Dreyfus**, M.D. (The University of Paris), 1986-1989.
Multileveled regulation of the human cholinesterase genes and their protein products.
INSERM exchange visitor. INSERM Fellow, Paris.

2. **Yaron Lifson-Lapidot**, M.D. (Ben-Gurion University), 1989-1991.
Elements of cholinergic signalling in Hematopoiesis.
Levi Eshkol Fellowship

3. **Daniel Grisaru**, M.D. (Tel Aviv University), 1996 – 2001.
Morphogenic Function(s) of Acetylcholinesterase variants and fragments thereof in normal and pathological development. Senior Lecturer, Tel-Aviv University.
(co-supervisor: A. Eldor, TAU). *Meirbaum award, 1998.*

4. **Asher Salmon**, M.D. (Technion), 1998 –.
Genetic Engineering approaches into cholinesterase interactions with Xenobiotic agents.
ENN short-term fellowship, 1998. Barclays Post-doctoral Fellowship, 2001.

5. **Chava Perry**, M.D. (HUJ), 2000-
Molecular mechanisms underlying acetylchoinesterase associated tumorogenesis.
(co-supervisor: the late A. Eldor, TAU)
Meirbaum Award, 2000.Long-term Ministry of Health Fellowship, 2001. Lecturer, Tel-Aviv University.

6. **Michael Levy**, M.D./Ph.D. program, (HUJ), 2001-
Antisense modulations of cognitive processes.
Co-Supervisor: Hagai Bergman (Faculty of Medicine). Interdisciplinary Center of Neuronal Computation Fellowship.

7. **Rinat Kahat**, M.D. (Technion), 2001-
Acetylcholinesterase in retinal functioning.
(co-supervisor with Ido Perlman, Rapaport Institute).

D. **M.D. Basic Research Fellows**

1. **Nissim Razon**, M.D., (Tel Aviv University) 1982.
Selective Impairment of Gene Expression in Human Brain Tumors.
Bornstein Award, 1982. Senior Lecturer, Tel Aviv University.

2. **Avi Matzkel**, M.D., (Tel Aviv University), 1983.
Polymorphism of acetylcholinesterase in fetal human tissues.

3. **Gustavo Malinger**, M.D., (Tel Aviv University), 1986.
The Expression of Human Cholinesterase Genes in Normal and Malignant ovary.
Israel Fertility Association Award, 1986.

4. **Eduardo Schejter**, M.D., (Tel Aviv University), 1987.
Expression of human cholinesterase genes in muscle.

5. **Ari Ayalon**, M.D., (Tel Aviv University), 1988.
Expression of human cholinesterase genes in fetal and neoplastic brain tissues as detected by in situ hybridization.

6. **Adrian Katz**, M.D., (Tel Aviv University), 1989.
Spermatogenic expression of human cholinesterase genes.

7. **Asher Salmon**, M.D., (Technion, Haifa), 1997.
Variable interactions of alternative cholinesterases with heroin derivatives.

8. **Tatiana Wender**, M.D., (Ben Gurion University), 2001.
ACHE promoter polymorphisms in occupationally induced Parkinson's Disease.

9. **Amir Dori**, M.D., PhD (Ben Gurion University), 2002.
Neurogenic Functions of Embryonic AChE.

E. **Post-Doctoral Fellows**

1. **Sherena Cedar**, Ph.D. (in Immunology, London University) 1983 - 1984.
The expression of cholinesterase in inducible human erythroleukaemic cells lines.
EMBO Post-Doctoral Fellowship.

2. **Catherine Prody**, Ph.D. (in Biochemistry, University of California, Berkeley) 1984 - 1988.
Molecular cloning of human cDNA sequences coding for cholinesterases.
MDA Fellow.

3. **Judy Lieman-Hurwitz**, Ph.D. (in Virology, Weizmann Institute) 1987-1989.
Expression of cloned acetylcholinesterase cDNA in microinjected *Xenopus* oocytes.
Levi Eshkol Fellowship.

4. **Lewis Neville**, Ph.D. (in Neurobiology, University of Southhampton) 1989-1991.
Expression of natural and site-directed cholinesterase variants in microinjected Xenopus oocytes.
Golda Meir Fellowship.

5. **Averell Gnatt**, Ph.D. (in Biochemistry, Hebrew University), 1991-1992.
site-directed mutagenesis of recombinant human butyrylcholinesterase.
Honorary ESN Lecturer, 1992, Dublin

6. **Rachel Karpel**, Ph.D., (in Ecology, Hebrew University), 1991 - 1994.

Cholinergic signalling and cell division control.
ICRF Fellowship.

7. **Mikael Schwarz**, Ph.D. (in Botany, Hebrew University), 1992- 1994.
Molecular dissection of catalytic events of cholinesterases.
Levy Eshkol Fellowship.

8. **Ellen Chaikin**, Ph.D. (in Developmental Biochemistry, Hebrew University), 1993.
Expression of cholinesterases in osteogenesis.
Golda Meir Fellowship.

9. **Christian Andres**, M.D., Ph.D (in Neurochemistry, University of Strasbourg), 1993 - 1995.
Transgenic expression of human acetylcholinesterase in murine nervous system.
INSERM & NCRD-Israel Ministry of Science Exchange Fellowships.
Professor of Genetics, University of Tours.

10. **Alon Friedman**, M.D., Ph.D. (in Neurobiology, Ben Gurion University), 1996-1998.
Electrophysiological and molecular mechanisms underlying cholinergic hyperexcitation.
Smith Psychobiology Post-doctoral Fellowship. Foulkes Prize, 1997. Teva Prize, 1997.
Senior Lecturer, Ben-Gurion University.

12. **Ron Broide**, Ph.D. (in Neurobiology, University of CA., Irvine), 1995-1997.
Photoreceptor degeneration in ACHE transgenic mice.
Valazzi - Pikovsky Fellowship.

13. **Christina Erb**, Ph.D. (in Pharmacology, University of Mainz), 1999-2000.
Transgenic approach to the molecular neuropharmacology of cholinergic transmission.
Long-Term Minerva Fellowship.

14. **Klara Birikh**, Ph.D. (in Molecular Biology, Moscow University), 1999 – 2000.
Conditional antisense suppression of neuronal acetylcholinesterase production.
Long-Term EMBO Fellowship. Senior Researcher, Chemiyakin Institute, Moscow.

15. **Cesar Flores Flores**, Ph.D. (in Biochemistry, Univ. of Murcia, Spain) 1999 – 2002
Combinatorial search for monoclonal anti-AChE antibodies.
Golda Meir Fellowship (declined); *Long-term FEBS Fellowship.*
Researcher, The University of Murcia, Spain.

16. **Amir Dori**, MD, PhD (in Physiology, BGU), 2002-2003.
Neurodevelopmental implications of acetylcholinesterase gene expression.
Smith Psychology Post-doctoral Fellowship.

17. **Cinthya Assuncao Guimaraes**, Ph.D. (in Neurogenetics, University Rio de Janeiro, Brazil)
EMBO New World Fellowship, 2002 – 2004.

18. **Yoram Ben-Shaul, PhD**. (in Computational Neuroscience, HUJ) 2003-2004
Interdisciplinary Center for Neural Computation Post Doctoral Fellowship 2003-2004

F. MAJOR EXTRAMURAL COLLABORATIONS

1. **Charles J. Arntzen,** Director,Arizona Biodesign Institute and Tsafrir Mor, Assistant Professor, Arizona State University, Member, US President's Advisory Council on Science.
Human acetylcholinesterase production in transgenic tomatos (DARPA grant 2001-2004).

2. **Fritz Eckstein,** The Max Planck Institut for Experimental Medicine, Gottingen, W. Germany.
Use of Antisense oligodeoxynucleotides for the *in vivo* modulation of cholinergic signalling and survival.
(Ministry of Science grant, 1991 - 1994; GIF grant, 1994-1997;
Joels Visiting Professor November 1997 - February 1998, Hebrew University of Jerusalem).

3. The late **Amiram Eldor**, Chairman, Department of Hematology, Sourasky Medical Center, Tel Aviv.
Hematopoietic function(s) of human acetylcholinesterase variants.

4. **Alon Friedman,** Department of Neurosurgery, Soroka Medical Center, Beersheva.
US Army Medical Research and Development Grant, 1999-2004.

5. **Israel Hanin**, Chairman, Department of Pharmacology & Experimental Therapeutics, Loyola University, Chicago. Cholinotoxic effects on brain-region specific alterations in G,C-rich transcripts.
Lady Davies Fellow, 1993. Smith Psychobiology Fund, 1993.

6. **James Patrick**, Vice President and Dean of Research, Baylor Medical School, Houston, Texas.
Transgenic modulations of cholinergic functions. (BSF Grants, 1989-1992, 1993-1996, 1997-1999, 2000-2002).

7. **Gene Robinson**, Dept. of Entomology, University of Illinois, Urbana, IL U.S.A.
Molecular Genetics approach to Honey Bee Acetylcholinesterase. *Fullbright Fellow,* 1996. *Smith Psychobiology Fund,* 1996.

8. **Raz Yirmiya**, Dept. of Psychology, HUJ.
Cholinergic modulations of mammalian behavior

9. **Haim Zakut**, The Sackler Faculty of Medicine, Tel Aviv University.

Expression of human cholinesterase genes in fetal development and in neoplastic tissues (Academy grant, 1994-1996: Ministry of health grant, 1995-1996).

PATENT APPLICATIONS

Title	Application/ Pat. No	Application Date	Current Status	Yissum (Luzz.)
Genetically Engineered Human Cholinesterases	IL Priority	21/03/89	Renewed 03/02/02	1961.00 (89703)
	EU 0 388 906	20/03/90	Granted 14/06/95	1961.03 (90105274)
	F 0 388 906	14/06/95	Granted 14/06/95	1961.04
	CH 0 388 906	14/06/95	Granted 14/06/95	1961.05
	GB 0 388 906	14/06/95	Granted 14/06/95	1961.06 (90105274.6)
	DE 690 20018.8	14/06/95	Granted 14/06/95	1961.07
	US 5,595,903.	02/08/93	Granted 21/01/97	1961.08 (6321) (08/111,314)
Transgenic Animal Assay System for Anticholinesterase Substances	US 5,932,780	09/01/95	Granted 03/08/1999	2098.01 (08/370, 156 [814095])
	PCT/US 95/02806	28/02/95	Published 31/8/95	2098.02 (6312)
	EU 95913580.7	28/02/95	Filed	2098.03
	US 6,025,183	06/03/97	Granted 15/2/00	2098.04
	US 09/310,638	12/5/99	Examined	2098.05
Therapies Utilizing Antisense Oligonucleotides and Butyryl-Cholinesterase Inhibitors	US	12/12/96	Provisional	(60/035,266)
A method and composition for enabling passage through the blood-brain barrier	PCT/US97/21696	20/11/97	Published 28/5/9	2151.02 (6439)
	US 6,258,780	20/11/97	Granted 10/7/01	2151.03
	IL 129990	20/11/97	Filed	2151.04
	AUS 53642/98	20/11/97	Granted 10/7/01	2151.05
	CAN 2272280	20/11/97	Filed	2151.06
	JAP 10-523989	20/11/97	Filed	2151.08
Method of Screening For Genetic Predisposition to Anticholinesterase Therapy	PCT/US9 00322	11/1/96	Published 18/7/96 WO96/21744	2207.01 (6785)
	US 08/370,204	09/01/95	Allowed partially appeal 13/10/99	2207.03
	CAN 2,209,683	11/1/96	Filed	2207.04
	JAP 8-521788	11/1/96	Published 24/11/98	2207.06
	US 5,807,671 Priority	9/1/95	Granted 15/9/98	2207.00
	US 6,326,139	09/01/95	Granted 4/12/2001	2207-05

Synthetic antisense oligodeoxynucleotides targeted to human ACHE (Title as US patent)	PCT/US 97/23598	12/12/97	Published 18/6/98 WO98/26062	2304.01 (6707)
	US 6,121,046	12/12/97	Granted 19/09/00	2304.02
	IL 130162	12/12/97	Filed	2304.03
	AU 727611	12/12/97	Granted 14/10/00	2304.04
	CAN 2,274,985	12/12/97	Filed	2304.05
	EP 0951536	12/12/97	Published 15/9/99	2304.06
	JP 10-527069	12/12/97	Filed	2304.07
	US 09/572,630 Cip		Examined	2304.08
Synthetic antisense oligodeoxynucleotides and pharmaceutical composition containing them	PCT/EP 93/00911	15/4/93	Published 28/10/93 WO93/21202	2042.01 (6692)
	JAP 517984/93	15/4/93	Abandoned	2042.02
	EP 0636137	15/4/93	Granted Validated in FR,DE &UK	2042.03
	US 5,891,725	1/12/94	Granted 6/4/99	2042.05
	CAN 2,118,235	15/4/93	Filed	2042.06
Cip title: *Synthetic antisense oligodeoxynucleotides targeted to ACHE*	US 6,110,740 Cip patent	2/5/98	Granted 29/8/00	2042.07
Title: *Deoxyoligonucleotides and pharmaceutical compositions containing the same*	IL 101600 (Priority)	15/4/92	Granted 30/5/00	2042.00
Methods and compositions for the treatment of injury to the central nervous system	PCT/US 98/04503	6/3/98	Published 11/9/98 WO98/39486	2325.01 (6720)
	AUS 64521/98	6/3/98	Abandoned	2325.02
	CAN 2,283,068	6/3/98	Filed	2325.03
	US 09/380532	6/3/98	Examined	2325.04
	EP 98910229.8		Abandoned	2325.05
Diagnostic uses of antibodies against AChE or C-terminal peptides thereof	IL 130225 Priority	31/05/99	Examined	2463.00 (8248)
	PCT/IL 00/00312	31/5/00	Published 2/12/00 WO00/73343	2463.01
	US	31/5/00		2463.02
	EU	31/5/00		2463.03
	CA	31/5/00		2463.04
	IL	31/5/00		2463.05
Pharmaceutical compositions comprising AChE AS-ODNs for the treatment of muscular and neuromuscular disorders	IL 132972	16/11/99	Filed	2428.00 (6711)
	PCT/IL 00/00763	16/11/00	Filed	2428.01
Human antibodies to specific AChE variants and their use as diagnostic agents for cholinergic neurodeterioration processes	IL 140071	4/12/00	Filed	2557.00 (12261)
	PCT/IL 01/00464	19/06/01	Filed	2557.01
Antisense oligonucleotide against human AChE and uses thereof	IL 143379	24/05/01	Filed	2584.00 (13122)
System and method for assaying drugs	US 60/247,970 PCT/IL01/01051	14/11/00 14/11/01	Exhausted filed	2547 (13825)
Acetylcholinesterase-derived peptides and uses thereof	US-2003-0036632-A1 (cip of PCT) PCT/IL00/00311 IL 130224 IL 131707	31/5/00 30/05/99 02/09/1999	Just filed	2356.03 (7811) 2356.02 2356.00 2356.01

Genetically engineered proteins having human cholinesterase activity	US 5,215,909 (cip App) EU 206 200	18/Jun/1986 18/06/1986	Granted 01/Jun/1993 Granted 23/09/1992	0011.05 0011.03
Hybrid transgenic mouse with accelerated onset of Alzheimer type amyloid plaques in brain (with Mayo Clinic)			Pre-filing	2622.00

LIST OF PUBLICATIONS

HERMONA SOREQ

BOOKS AND MONOGRAPHS

2004 Meshorer, E. and Soreq, H.
Stress Hour: Towards the Molecular Biology of Stress Responses.
The United Kibbutz Publishers, Jerusalem (Hebrew).
Silman, I. Soreq, H. Fischer, A. Anglister, L. and Michaelson, D., Eds.
Cholinergic Mechanisms.
Martin Dunitz, London

1996 Seidman S. and Soreq, H.
Transgenic *Xenopus:* Microinjection Methods and
Developmental Neurobiology
Humana Press, Neuromethods vol. 28. A. Boulton and G.B. Baker, Series Eds. 225p.

1993 Soreq, H. and Zakut, H.
Human Cholinesterases and anticholinesterases.
Academic Press, San Diego 300 p.

1990 Soreq, H. and Zakut, H.
Cholinesterase genes: Multileveled regulation
Monographs in Human Genetics, Vol. 13
Karger, Basel (R.S. Sparkes, ed.) 120 p.

PEER REVIEWED RESEARCH PAPERS

1. Soreq, H. and Kaplan, R. (1971) Inducible and constitutive beta-galactosidase formation in cells recovering from protein synthesis inhibition. J. Bacteriol. **108**, 1147-1153.
2. Soreq, H., Nudel, U., Salomon, R., Revel, M. and Littauer, U. Z. (1974) *In vitro* translation of polyadenylic acid-free rabbit globin messenger RNA. J. Mol. Biol. **88**, 233-245.
3. Huez, G., Marbaix, G., Hubert, E., Leclercq, M., Nudel, U., Soreq, H., Salomon, R., Lebleux, B., Revel, M. and Littauer, U. Z. (1974) Role of polyadenylate segment in the translation of globin messenger RNA in *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA **71**, 3143-3146.
4. Marbaix, G., Huez, G., Burny, A., Cleuter, Y., Hubert, E., Leclercq, M., Chantrenne, H., Soreq, H., Nudel, U. and Littauer, U. Z. (1975) Absence of polyadenylate segment in globin messenger RNA accelerates its degradation in *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA **72**, 3065-3067.
5. Huez, G., Marbaix, G., Hubert, E., Cleuter, Y., Leclercq, M., Chantrenne, H., Devos. R., Soreq, H., Nudel, U. and Littauer, U. Z. (1975) Readenylation of polyadenylate-free globin messenger RNA restores its stability *in vivo*. Eur. J. Biochem. **59**, 589-592.
6. Nudel, U., Soreq, H., Littauer, U. Z., Huez, G., Marbaix, G., Hubert, E. and Leclercq, M. (1976) Globin mRNA species containing poly(A) segments of different lengths: Their functional stability in *Xenopus* oocytes. Eur. J. Biochem. **64**, 115-121.
7. Salomon, R., Sela, I., Soreq, H., Giveon, D. and Littauer, U. Z. (1976) Enzymatic acylation of hystidine to tobacco mosaic virus RNA. Virology **71**, 74-84.
8. Huez, G., Marbaix, G., Burny, A., Hubert, E., Leclercq, M., Cleuter, Y., Chantrenne, H., Soreq, H. and Littauer, U. Z. (1977). Degradation of deadenylated rabbit alpha-globin mRNA in *Xenopus* oocytes is associated with its translation. Nature **66**, 472-473.
9. Soreq, H. and Littauer, U. Z. (1977) Purifcation and characterization of polynucleotide phosphorylase from *E. coli*: Probe for the analysis of 3'-end sequences of messenger RNA. J. Biol. Chem. **252**, 6885-6888.
10. Grosfeld, H., Soreq, H. and Littauer, U. Z. (1977) Membrane-associated cytoplasmic mRNA in *Artemia salina*: Functional and physical changes during development. Nuc. Acids Res. **4**, 2109-2121.
11. Gilboa, E., Soreq, H. and Aviv, H. (1977) Initiation of RNA synthesis in isolated nuclei. Eur. J. Biochem. **77**, 393-400.
12. Salomon, R., Bar-Joseph, M., Soreq, H., Gozes, I. and Littauer, U. Z. (1978) Translation *in vitro* of Carnation mottle virus RNA: Regulatory function of the 3'-region. Virology **90**, 288-298.
13. Sehgal, P., Soreq, H. and Tamm, I. (1978) Does 3'-terminal poly(A) stabilize human fibroblast interferon mRNA in oocytes of *Xenopus laevis*? Proc. Natl. Acad. Sci. USA **75**, 5030-5033.
14. Kaempfer, R., Hollender, R., Soreq, H. and Nudel, U. (1979) Recognition of messenger RNA in eukaryotic protein synthesis: Equilibrium studies of the interaction between mRNA and the initiation factor that binds methionyl tRNAmet^{f}. Eur. J. Biochem. **94**, 591-600.
15. Soreq, H., Harpold, M. M., Evans, R. M., Darnell, J. E. and Bancroft, F. C. (1979) Rat growth-hormone gene: intervening sequences within the coding region. Nuc. Acids Res. **6**, 2471-2482.

16. Soreq, H., Harpold, M. M. and Darnell, J. E. (1980) Rate of synthesis and concentration of specific cloned mRNA sequences in cultured Chinese hamster ovary cells compared to liver cells. Biochem. Biophys. Res. Commun. 92, 485-491.

17. Weissenbach, J., Cherjanovsky, Y., Zeevi, M., Shulman, L., Soreq, H., Nir, U., Wallach, D., Perricaudet, M., Tiollais, P. and Revel, M. (1980) Two interferon mRNAs in human fibrobalsts: *In vitro* translation and *E. coli* cloning studies. Proc. Natl. Acad. Sci. USA 77, 7152-7156.

18. Soreq, H., Sagar, H. and Sehgal, P. B. (1981) Translational activity and functional stability of human fibroblast $beta_1$ and $beta_2$ interferon mRNA species lacking 3'-terminal sequences. Proc. Natl. Acad. Sci. USA 78, 1741-1745.

19. Soreq, H. and Miskin, R. (1981) Plasminogen activator in the developing rodent brain. Brain research 216, 361-374.

20. Miskin, R. and Soreq, H. (1981) Microinjected *Xenopus* oocytes synthesize active human plasminogen activator. Nuc. A. Res. 9, 3355-3364.

21. Soreq, H. and Miskin, R. (1981) Secreted proteins in the medium of microinjected *Xenopus* oocytes are degraded by oocyte proteases. FEBS Lett. 128, 305-310.

22. Miskin, R. and Soreq, H. (1981) Sensitive autoradiographic quantification of electrophoretically separated proteases. Analytical Biochem. 118, 252-258.

23. Soreq, H., Safran, A. and Zisling, R. (1982) Variations in gene expression during development of the rat cerebellum. Dev. Br. Res. 3, 65-79.

24. Eliyahu, D. and Soreq, H. (1982) Degranulation of rat cerebellum induces selective variations in gene expression. J. Neurochem. 38, 313-321.

25. Soreq, H., Parvari, R. and Silman, I. (1982) Biosynthesis and secretion of active acetylcholinesterase in *Xenopus* oocytes microinjected with mRNA from rat brain and from *Torpedo* electric organ. Proc. Natl. Acad. Sci. USA 79, 830-834.

26. Soreq, H., Bartfeld, D., Parvari, R. and Fuchs, S. (1982) Increase in the translatable mRNA for acetylcholine receptor during embryonic development of *Torpedo ocellata* electric organ. FEBS Lett. 139, 32-36.

27. Soreq, H., Gurwitz, D., Eliyahu, D. and Sokolovsky, M. (1982) Altered ontogenesis of muscarinic receptors in agranular cerebellar cortex. J. Neurochem. 39, 756-763.

28. Zisapel, N., Miskin, R., Laudon, M. and Soreq, H. (1982) Plasminogen activator is enriched in the synpatosomal plasma membrane. Brain Research 248, 129-139.

29. Soreq, H., Miskin, R., Zutra, A. and Littauer, U. Z. (1983) Modulation in the levels and localization of plasminogen activator in differentiating neuroblastoma cells. Dev. Brain Res. 7, 257-269.

30. Soreq, H., Safran, A. and Eliyahu, D. (1983) Modified composition of major ontogenetically regulated mRNAs and proteins in the cerebellum of old mice and of staggerer mutants, Dev. Brain Res. 10, 73-82.

31. Malnick, S. D. H., Shaer, A., Soreq, H. and Kaye, A. M. (1983) Estrogen-induced creatine kinase in the reproductive system of the immature female rat. Endocrinology 113, 1907-1909.

32. Parvari, R., Pecht, I. and Soreq, H. (1983) A microfluorometric assay for cholinestrases, sutiable for multiple kinetic determinations of picomoles of released thiocholine. Anal. Biochem. 133, 450-456.

33. Koren, R., Burstein, Y. and Soreq, H. (1983) Synthetic leader peptide modulates secretion of proteins from microinjected *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA 80, 7205-7210.

34. Soreq, H. and Miskin, R. (1983) Plasminogen activator in the developing rat cerebellum: Biosynthesis and localization in granular neurons. Dev. Brain Res. 11, 149-158.

35. Libermann, T. A., Razon, N., Bartal, A. D., Yarden, Y., Schlessinger, J. and Soreq, H. (1984) Expression of epidermal growth factor receptors in human brain tumors. Cancer Res. 44, 753-760.

36. Gurwitz, D., Razon, N., Sokolovsky, M. and Soreq, H. (1984) Expression of muscarinic receptors in primary brain tumors. Dev. Brain Res. 14, 61-71.

37. Soreq, H., Zevin-Sonkin, D. and Razon, N. (1984) Expression of cholinesterase gene(s) in human brain tissues: translational evidence for multiple mRNA species. The EMBO J. 3, 1371-1375.

38. Razon, N., Soreq, H., Roth, E., Bartal, A. and Silman, I. (1984) Characterization of levels and forms of cholinesterases in human primary brain tumors. Experimental Neurology 84, 681-695.

39. Burmeister, M., Avivi, A., Schlesinger, J. and Soreq, H. (1984) The biosynthesis of EGF-containing polypeptides in mRNA-microinjected *Xenopus* oocytes. The EMBO Journal 3. 1499-1505.

40. Soreq, H., Zevin-Sonkin, D., Avni, A., Hall, L. and Spierer, P. (1985) A human acetylcholinesterase gene identified by homology to the *Drosophila* gene. Proc. Natl. Acad. Sci. USA 82, 1827-1831.

41. Libermann, T. A., Nusbaum, H. R., Razon, N., Kris, R., Lax, I., Soreq, H. Whittle, N., Waterfield, M. D., Ullrich, A. and Schlessinger, J. (1984) Amplification, enhanced expression and possible rearrangement of the EGF receptor gene in primary human brain tumors of glial origin. Nature 313, 144-147.

42. Zakut, H., Matzkel, A., Schejter, E., Avni, A. and Soreq, H. (1985) Polymorphism of acetylcholinesterase in discrete regions of the developing fetal human brain. J. Neurochem. 45, 382-389.

43. Zevin-Sonkin, D., Avni, A., Zisling, R., Koch, R. and Soreq, H. (1985) Expression of acetylcholinesterase gene(s) in the human brain: Molecular cloning evidence for cross-homologous sequences. J. Physiol. (Paris) 80, 221-228.

44. Dziegielewska, K. M., Saunders, N. R. and Soreq, H. (1985) Messenger RNA from developing rat cerebellum directs *in vitro* biosynthesis of plasma proteins. Dev. Brain Res. 23, 259-267.

45. Libermann, T. A., Nusbaum, H. R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M. D., Ullrich, R. and Schlessinger, J. (1985) Amplification and overexpression of the EGF receptor gene in primary human glioblastomas. J. Cell Science 3, 161-172.

46. Egozi, Y., Sokolovsky, M., Schejter, E., Blatt, I., Zakut, H., Matzkel, A. and Soreq, H. (1986) Divergent regulation of muscarinic binding sites and acetylcholinesterase in discrete regions of the developing human fetal brain. Molecular Neurobiol. 6, 55-70.

47. Dziegielewska, K. M., Saunders, N. R., Schejter, E. J., Zakut, H., Zevin-Sonkin, D., Zisling, R. and Soreq, H. (1986) Synthesis of plasma proteins in fetal, adult and neoplastic human brain tissue. Dev. Biol. 115, 93-104.

48. Prody, C., Zevin-Sonkin, D., Gnatt, A., Koch, R., Zisling, R., Goldberg, O. and Soreq, H. (1986) Use of synthetic oligodeoxynucleotide probes for the isolation of a human cholinesterase cDNA clone. J. Neurosci. Res. 16, 25-36.

49. Soreq, H., Dziegielewska, K. M., Zevin-Sonkin, D. and Zakut, H. (1986) The use of mRNA translation *in vitro* and *in ovo* followed by crossed immunoelectrophoretic autoradiography to study the biosynthesis of human cholinesterases. Cell Molec. Neurobiol. 6, 227-237.

50. Prody, C. A., Zevin-Sonkin, D., Gnatt, A., Goldberg, O. and Soreq, H. (1987) Isolation and characterization of full-length cDNA clones coding for cholinesterase from fetal human tissues. Proc. Natl. Acad. Sci. USA 84, 3555-3559.

51. Soreq, H. (1987) The case of the human pink spider toxinase.

Trends in Biotechnology 5, 183-185.

52. Soreq, H., Zamir, R., Zevin-Sonkin, D. and Zakut, H. (1987) Human cholinesterase genes localized by hybridization to chromosomes 3 and 16. Human Genetics, 77, 325-328.

53. Soreq, H., Malinger, G. and Zakut, H. (1987) Expression of cholinesterase genes in developing human oocytes revealed by *in situ* hybridization. Human Reproduction 2, 689-693.

54. Zakut, H., Even, L., Birkenfeld, S., Malinger, G., Zisling, R. and Soreq, H. (1988) Modified properties of serum cholinesterases in primary carcinomas. Cancer, 61, 727-737.

55. Mollgard, K., Djiegielewska, K. M., Saunders, N. R., Zakut, H. and Soreq, H. (1988) Synthesis and localization of plasma proteins in the developing human brain: Integrity of the fetal blood-brain barrier to endogenous proteins of hepatic origin. Dev. Biol., 128, 207-221.

56. Livneh, A., Sarova, I., Michaeli, D., Pras, M., Wagner, K., Zakut, H. and Soreq, H. (1988) Antibodies against acetylcholinesterase and low levels of cholinesterases in a patient with an atypical neuromuscular disorder. Clinical Immunology and Immunopathology, 48, 119-131.

57. Farkash, Y., Soreq, H. and Orly, Y. (1988) Biosynthesis of catalytically active rat testosterone 5-reductase in microinjected *Xenopus* oocytes: Evidence for tissue- specific differences in translatable mRNA. Proc. Natl. Acad. Sci. USA, 85, 5824-5828.

58. Dreyfus, P., Zevin-Sonkin, D., Seidman, S., Prody, C., Zisling, R., Zakut, H. and Soreq, H. (1988) Cross homologies and structural differences between human cholinesterases revealed by antibodies against cDNA-produced human butyrylcholinesterase peptides. J. Neurochem. 51, 1858-1867.

59. Malinger, G., Zakut, H. and Soreq, H. (1989) Cholinoceptive properties of human primordial, pre-antral and antral oocytes: *In situ* hybridization and biochemical evidence for expression of cholinesterase genes. J. Molec. Neuroscience, 1, 77-84.

60. Prody, A. C., Dreyfus, P., Zamir, R., Zakut, H. and Soreq, H. (1989) *De novo* amplification within a "silent" human cholinesterase gene in a family subjected to prolonged exposure to organophosphorous insecticides. Proc. Natl. Acad. Sci. USA. 86, 690-694.

61. Zakut, H., Zamir, R., Sindel, L. and Soreq, H. (1989) Mapping genes on chorionic villi chromosomes by hybridization *in situ*. Harefuah, 39, 81-85 (Hebrew).

62. Soreq, H., Seidman, S., Dreyfus, P. A., Zevin-Sonkin, D. and Zakut, H. (1989) Expression and tissue-specific assembly of human butyrylcholinesterase in microinjected *Xenopus* oocytes. J. Biol. Chem., 264, 10608-10613.

63. Lifson-Lapidot, Y., Prody, C. A., Ginzberg, D., Meytes, D., Zakut, H. and Soreq, H. (1989) Co-amplification of human acetylcholinesterase and butyryl-cholinesterase genes in blood cells: Correlation with various leukemias and abnormal megakaryocytopoiesis. Proc. Natl. Acad. Sci. USA, **86**, 4715-4719.

64. Dreyfus, P. A., Seidman, S., Pincon-Raymond, M. Murawsky, M., Rieger, F., Schejter, E., Zakut, H. and Soreq, H. (1988) Tissue-specific processing and polarized compartmentalization of nascent cholinesterase in microinjected *Xenopus* oocytes. Cell. Molec. Neurobiol. **9**, 323-341.

65. Ludgate, M., Dong, P., Dreyfus, P. A., Zakut, H., Taylor, P., Vassart, G. and Soreq, H. (1989) Definition, at the molecular level, of a thyroglobulin-acetyl-cholinesterase shared epitope: Study of its pathophysiological significance in pateints with Grave's ophthalmopathy. Autoimmunity, **3**, 167-176.

66. Benvenisty, N., Shoshani, T., Farkash, Y., Soreq, H. and Reshef, L. (1989) Trans-activation of phosphoenolpyruvate carboxykinase (GTP) gene expression by rat liver mRNA in microinjected *Xenopus* oocytes. Molec. Cell. Biol., **9**, 5244-5247

67. Zakut, H., Zamir, R., Sindel, L. and Soreq, H. (1989) Gene mapping on chorionic villi chromosomes by hybridization *in situ* refinement of cholinesterase genes localization to chromosomes 3q21, 3q26-ter and 16q-21. Human Reproduction, **4**, 941-946.

68. Ballas, N., Broido, S., Soreq, H. and Loyter, A. (1989) Efficient functioning of plant promoters and poly(A)sites in *Xenopus* oocytes. Nuc. Acids Res., **17**, 7891-7983.

69. Gnatt, A., Prody, C.A., Zamir, R., Lieman-Hurwitz, J., Zakut, H. and Soreq, H. (1990) Expression of alternatively terminated unusual CHEmRNA transcripts mapping to chromosome 3q26-ter in nervous system tumors. Cancer Research, **50**, 1983-1987.

70. Ben Aziz, R. and Soreq, H. (1990) Improving poor *in vitro* transcription from G,C-rich genes. Nucleic Acids Research. **18**, 3418.

71. Zakut, H., Ehrlich, G., Ayalon, A., Prody, C. A., Malinger, G., Seidman, S., Kehlenbach, R., Ginzberg, D. and Soreq, H. (1990) Acetylcholinesterase and butyrylcholinesterase genes co-amplify in primary ovarian carcinomas. J. Clin. Invest., **86**, 900-908.

72. Patinkin, D., Seidman, S., Eckstein, F., Benseler, F., Zakut, H. and Soreq, H. (1990) Manipulations of cholinesterase gene expression modulate Murine Megakaryocytopoiesis *in vitro*, Molec. Cell. Biol., **10**, 6046-6050.

73. Neville, L.F., Gnatt, A., Padan, R., Seidman, S. and Soreq, H. (1990) Anionic site interactions in human butyrylcholinesterase disrupted by two adjacent single point mutations. J. Biol. Chem., **265**, 20735-20739.

74. Soreq, H., Ben-Aziz, R., Prody, C.A., Gnatt, A., Neville, L., Lieman-Hurwitz, J., Lev-Lehman, E., Ginzberg, D., Seidman, S. Lapidot-Lifson, Y. and Zakut, H. (1990). Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G,C-rich attenuating structure. Proc. Natl. Acad. Sci. USA, **87**, 9688-9692.

75. Neville, L.F., Gnatt, A., Lowenstein, Y. and Soreq, H. (1990) Aspartate 70 to glycine substitution confers resistance to naturally occurring and synthetic anionic site ligands on *in ovo* produced human butyrylcholinesterase. J. Neurosci. Res., **27**, 452-460.

76. Soreq, H., Katz, A., Richler, C., Malinger, G. and Zakut, H. (1990) Expression of cholinesterase genes in human germline cells: implications on reproduction. Is. J. of Obstetrics and Gynecology, **1**, 111-113.

77. Soreq, H. (1991) Cholinesterases in cellular and molecular neurobiology. Cell Molec. Neurobiol. **11**, 3-5.

78. Gnatt, A., Ginzberg, D., Lieman-Hurwitz, J., Zamir, R., Zakut, H. and Soreq, H. (1991) Human acetylcholinesterase and butyrylcholinesterase are encoded by two distinct genes. Cell. Molec. Neurobiol., **11**, 91-104.

79. Velan, B., Kronman, C., Grosfeld, H., Leitner, M., Gozes, Y., Flashner, Y., Sery, T., Cohen, S., Ben-Aziz, R., Seideman, S., Shafferman, A. and Soreq, H. (1991) Recombinant human acetylcholinesterase is secreted from transiently transfected 293 cells as a soluble globular enzyme. Cell. Molec. Neurobiol., 11, 143-156.

80. Zakut, H., Lieman-Hurwitz, J., Zamir, R., Sindell, L., Ginzberg, D. and Soreq, H. (1991) Chorionic villi cDNA Library displays expression of butyrylcholinesterase: putative genetic disposition for ecological danger. Prenatal Diagnosis, **11**, 597-607.

81. Lapidot-Lifson, Y., Patinkin, D., Prody, C.A., Ehrlich, G., Seidman, S., Ben-Aziz, R., Benseler, F., Eckstein, F., Zakut, H. and Soreq, H. (1992) Cloning and antisense oligodeoxynucleotide inhibition of a human homolog of cdc_2 required in hematopoiesis. Proc. Natl. Acad. Sci. USA, **89**, 579-583.

82. Neville, L.F., Gnatt, A., Loewenstein, Y., Seideman, S., Ehrlich, G. and Soreq, H. (1992) Intramolecular relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BCHE. EMBO J., 11, 1641-1649.

83. Zakut, H., Lapidot-Lifson, Y., Beeri R., Ballin, A. and Soreq, H. (1992) In vivo gene amplification in non-cancerous cells: Cholinesterase genes and oncogenes amplify in thrombocytopenia associated with lupus erythematosus. Mutation Research, **276**, 275-284.

84. Soreq, H., Meir, R. and Zakut, H. (1992) (Colinesterase inhibitors: medical and structural implications of their mechanism of action) Harefuah, 123, 100-110.

85. Ehrlich, G., Viegas-Pequignot, E., Ginzberg, D., Sindel, L., Soreq, H. and Zakut, H. (1992). Mapping the human acetylcholinesterase gene to chromosomes 7q22 by fluorescent *in situ* hybridization coupled with selective PCR amplification from a somatic hybrid cell panel and chromosome-sorted DNA libraries. Genomics, 13, 1192-1197.

86. Soreq, H., Gnatt, A., Loewenstein, Y. and Neville, L.F. (1992) Excavations into the active site gorge of cholinesterases. Trends in Biochem. Sci., 17, 353-358.

87. Richler, C., Soreq, H. and Wahrman, J. (1992) X inactivation in mammalian testis is correlated with inactive X-specific transcription. Nature Genetics 2, 192-195 (accompanied by a News and Views cover, p. 169-170).

88. Ben Aziz-Aloya, R., Seidman, S., Timberg, R., Sternfeld, M., Zakut, H. and Soreq, H. (1993) Expression of a human acetylcholinesterase promoter-reporter construct in developing neuromuscular junctions of *Xenopus* embryos. Proc. Natl. Acad. Sci. U.S.A., 90, 2471-2475.

89. Loewenstein, Y., Denarie, M., Zakut, H. and Soreq, H. (1993) Molecular dissection of cholinesterase domains responsible for carbamate toxicity. Chemical-Biological Interactions 87:209-216.

90. Loewenstein, Y., Gnatt, A., Neville, L.F. and Soreq, H. (1993) A chimeric human cholinesterase: identification of interaction sites responsible for sensitivity to acetyl or butyrylcholinesterase-specific ligands. J. Mol. Biol, 234:289-296.

91. Lev-Lehman, E., Hornreich, G., Ginzberg, D., Gnatt, A., Meshorer, A., Eckstein, F., Soreq, H. and Zakut, H. (1994) Antisense inhibition of acetylcholinesterase gene expression causes transient hematopoietic alterations *in vivo*. Gene Therapy 1:127-135.

92. Loewenstein, Y., Gnatt, A., Neville, L.F., Zakut, H. and Soreq, H. (1993) Structure-function relationship studies in human cholinesterases reveal genomic origins for individual variations in cholinergic drug responses. Prog. Neuro-Psychopharmacol. Biol. Psychiat. 17, 905-926.

93. Karpel, R., Ben Aziz-Aloya, R., Sternfeld, M., Ehrlich, G., Ginzberg, D., Tarroni, P., Clementi, F., Zakut, H. and Soreq, H. (1994) Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. Exptl. Cell Res. 210, 268-277.

94. Gnatt, A., Loewenstein, Y., Yaron, A., Schwarz, M. and Soreq, H. (1994) Site-directed mutagenesis of active site residues reveals plasticity of human butyrylcholinesterase in substrate and inhibitor interactions. J. Neurochem., 62, 749-755.

95. Seidman, S., Ben-Aziz Aloya, R., Timberg, R., Loewenstein, Y., Velan, B., Shafferman, A., Liao, J., Norgaard-Pedersen, B., Brodbeck, U. and Soreq, H. (1994) Overexpressed monomeric human acetylcholinesterase induces subtle ultrastructural modifications in developing neuromuscular junctions of *Xenopus laevis* embryos. J. Neurochem., 62, 1670-1681.

96. Beeri, R., Gnatt, A., Lapidot-Lifson, Y., Ginzberg, D., Shani, M., Zakut, H. and Soreq, H. (1994) Testicular Amplification and impaired transmission of human butrylcholinesterase cDNA in transgenic mice. Human Reproduction 9, 284-292.

97. Ehrlich, G., Ginzberg, D., Loewenstein, Y., Glick, D., Kerem, B., Ben-Ari, S., Zakut, H. and Soreq, H. (1994) Population diversity and distinct haplotype frequencies associated with ACHE and BCHE genes of Israeli Jews from Trans-Caucasian Georgia and from Europe. Genomics 22 288-295.

98. Ehrlich, G., Patinkin, D., Ginzberg, D., Zakut, H., Eckstein, F. and Soreq, H. (1994) Use of partially phosphorothioated "antisense" oligodeoxynucleotides for sequence-dependent modulation of hematopoiesis in culture. Antisense Research and Development, 4, 173-183.

99. Soreq, H., Ehrlich, G., Schwarz, M., Loewenstein, Y., Glick, D. and Zakut, H. (1994) Mutations and impaired expression in the Human ACHE and BCHE genes: Neurological implications. Biomedicine and Pharmacotherapeutics 48, 253-259.

100. Soreq, H., Patinkin, D., Lev-Lehman, E., Grifman, M., Ginzberg, D., Eckstein, F. and Zakut, H. (1994) Antisense oligonucleotide inhibition of acetylcholinesterase gene expression induces progenitor cell expansion and suppresses hematopoietic apoptosis *ex vivo*. Proc. Natl. Acad. Sci. USA, 91, 7907-7911.

101. Shapira, M., Seidman, S., Sternfeld, M., Timberg, R., Kaufer, D., Patrick, J.W. and Soreq, H. (1994) Transgenic engineering of neuromuscular junctions in *Xenopus laevis* embryos transiently overexpressing key cholinergic proteins. Proc. Natl. Acad. Sci. USA, 91, 9072-9076.

102. Patinkin, D., Lev-Lehman, E., Zakut, H., Eckstein, F. and Soreq, H. (1994) Antisense inhibition of butyrylcholinesterase gene expression predicts adverse hematopoietic consequences to cholinesterase inhibitors. Cell. Molec. Neurobiol. 14:459-474.

103. Dobianer, K., Hruza, Ch., Ehrlich, G., Sevelda, P., Czerwenka, K., Soreq, H., Spona, J. and Zakut, H. (1995) HER-2 amplification but not butyrylcholinesterase mutability reflects agressiveness of European-originated ovarian tumors. Gynecol. Oncol., 56, 200-206.

104. Lev-Lehman, E., El-Tamer, A., Yaron, A., Grifman, M., Ginzberg, D., Hanin, I. and Soreq, H (1995) Cholinotoxic effects on acetylcholinesterase gene expression are associated with brain-region specific alterations in G,C-rich transcripts. Brain Res. 661,75-82.

105. Schwarz, M., Loewenstein-Lichtenstein, Y., Glick, D., Liao, J., Norgaard-Pedersen, B. and Soreq, H. (1995) Successive organophosphate inhibition and oxime reactivation reveals distinct responses of recombinant human cholinesterase variants. Molec. Brain Res. 31, 101-110.

106. Seidman, S., Sternfeld, M., Ben Aziz-Aloya, R., Timberg, R., Kaufer-Nachum, D. and Soreq, H. (1995) Synaptic and epidermal accumulations of human acetylcholinesterase is encoded by alternative 3'-terminal exons. Mol. Cell. Biol. 15, 2993-3002.

107. Beeri, R., Andres, C., Lev-Lehman, E., Timberg, R., Huberman, T., Shani, M. and Soreq, H. (1995) Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. Curr. Biol., 5, 1063-1071.

108. Loewenstein-Lichtenstein, Y., Schwarz, M., Glick, D., Norgaard-Pederson, B., Zakut, H. and Soreq, H. (1995) Genetic predisposition to adverse consequences of anti-cholinesterases in "atypical" BCHE carriers. Nature Medicine, 1, 1082-1085.

109. Soreq, H. (1995) Cholinergic symptoms and Gulf War Syndrome. Nature/Medicine 1, 1226 (correspondence).

110. Karpel, R., Sternfeld, M., Ginzberg, D., Guhl, E., Graessman, A. and Soreq H. (1996) Overexpression of alternative human acetylcholinesterase forms modulates process extensions in cultured glioma cells. J. Neurochem., 66, 114-123.

111. Nechushtan, H., Soreq, H., Kuperstein, V., Tshori, S. and Razin, E. (1996) Murine mast cells express acetylcholinesterase. FEBS Lett., 379:1-6.

112. Grifman, M., Lev-Lehman, E., El-Tamer, A., Ginzberg, D., Hanin, I. and Soreq, H. (1996) Differential display of mRNA transcripts in the nervous system: *ex vivo* and *in vivo* studies. Neuroscience Protocols, 96-020-05-01-11.

113. Friedman, A., Kaufer-Nachum, D., Shemer, J., Hendler, I., Soreq, H. and Tur-Kaspa, I. (1996). Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nature Medicine 2:1382-1385. Accompanied by: News&Views: The Gulf War, Stress and a leaky blood-brain barrier. Israel Hanin. Nature Medicine. 2:1307-1308.

114. Loewenstein-Lichtenstein, Y., Glick, D., Gluzman, N., Sternfeld, M., Zakut, H. and Soreq, H. (1996). Overlapping drug interaction sites of human butyrylcholinesterase dissected by site-directed mutagenesis.Mol.Pharmacol. 50, 1423-1431.

115. Broide, R., Grifman, M., Shapira, M., Ginzberg, D. and Soreq, H. (1997) Genetic manipulations of cholinergic communication reveal trans-acting control mechanisms over acetylcholine receptors. J. Receptor Res., 17:279-291.

116. Birikh, K., Berlin, U.A., Soreq, H. and Eckstein, F. (1997) Probing accessible sites for ribozymes on human acetylcholinesterase RNA. RNA, 4, 429-437.

117. Soreq, H., Kaufer, D. and Friedman, A. (1997) Less stress - more pressure? Nature Medicine 2, 366 (correspondence).

118. Lidar, T., Andres, C., Yakar, S., Langevitz, P., Zeilig, G., Ohry, A., Bakimer, R., Soreq H. and Livneh A. (1997) Clinically insignificant (natural) autoantibodies against acetylcholinesterase in the sera of patients with a variety of neurologic, muscular and autuimmune diseases. Immunolgy Letters 55, 79-84.

119. Lev-Lehman, E., Deutsch, V., Eldor, A. and Soreq, H. (1997). Immature human megakaryocytes produce nuclear-associated acetylcholinesterase. Blood, 89, 3644-3653.

120. Sternfeld, M., Rachmilewitz, J., Loewenstin-Lichtenstein, Y., Andres, C., Timberg, R., Ben-Ari, S., Soreq, H. and Zakut, H. (1997). Normal and "Atypical" Butyrylcholinesterase in placental development, function and malfunction. Cell.Molec.Neurobiol. 17, 315-332.

121. Andres, C., Beeri, R., Friedman, A., Lev-Lehman, E., Henis, S., Timberg, R., Shani, M. and Soreq, H. (1997). ACHE transgenic mice display embryonic modulations in spinal cord CHAT and neurexin Iβ gene expression followed by late-onset neuromotor deterioration. Proc. Natl. Acad. Sci. USA 94, 8173-8178.

122. Grifman, M. and Soreq, H. (1997). Differentiation intensifies the susceptibility of phaeochromocytoma cells to antisense oligodeoxynucleotide-dependent suppression of acetylcholinesterase activity. Antisense Research and Nucleic Acids Drug Development 7, 351-359.

123. Beeri, R., LeNovere, N., Mervis, R., Huberman, T., Grauer, E., Changeux, J.P. and Soreq, H. (1997). Enhanced hemicholinium binding and attenuated dendrite branching in cognitively impaired ACHE-transgenic mice. J. Neurochem. 69, 2441-2451.

124. Grifman, M., Arbel, A., Ginzberg, D., Glick, D., Elgavish, S., Shaanan, B. and Soreq, H. (1997). Acetylcholinesterase phoshorylation at non-consensus protein kinase A sites enhances the rate of acetylcholine hydrolysis. Molecular Brain Res., 51, 179-187.

125. Sternfeld, M., Ming, G-L., Song, H-J., Sela, K., Poo, M-M. and Soreq, H. (1998). Acetylcholinesterase enhances neurite growth and synapse development through alternate contributions of its hydrolytic capacity, core protein and variable C-termini. J. Neurosci. **18**, 1240-1249.

126. Kaufer, D., Friedman, A., Seidman, S. and Soreq, H. (1998). Acute stress facilitates long-lasting changes in cholinergic gene expression. Nature **393**, 373-377. Accompanied by: News & Views: The stress of Gulf War Syndrome. Sapolsky, R.M. Nature, **393**:308-309.

127. Andres, C., Seidman, S., Beeri, R., Timberg, R. and Soreq, H. (1998) Transgenic acetylcholinesterase induces enlargement of murine neuromuscular junctions but leaves spinal cord synapses unchanged. Neurochem. Int. **32**:449-456.

128. Sternfeld, M., Patrick, J.D. and Soreq, H. (1998). Position effect variegations and brain-specific silencing in transgenic mice overexpressing human acetylcholinesterase variants. J. Physiol (Paris) **92**, 249-256.

129. Friedman, A., Kaufer, D., Pavlovsky, L. and Soreq, H. (1998). Cholinergic activation induces activity-dependent electrophysiological and transcriptional responses in hippocampal slices. J. Physiol. (Paris) **92**, 329-336.

130. Grifman, M., Galyam, N., Seidman, S. and Soreq, H. (1998). Functional redundancy of acetylcholinesterase and neuroligin in mammalian neuritogenesis. Proc. Natl. Acad. Sci. USA **95**, 13935-13940.

131. Grisaru, D., Lev-Lehman, E., Shapira, M., Chaikin, E., Lessing, J.B., Eldor, A., Eckstein, F. and Soreq, H. (1999) Human osteogenesis involves differentiation-dependent increases in the morphogenically-active 3' alternative splicing variant of acetylcholinesterase. Molec. Cell. Biol. **19**, 788-795.

132. Shapira, M., Seidman, S., Livny, N. and Soreq, H. (1998) *In vivo* and *in vitro* resistance to multiple anticholinesterases in *Xenopus laevis* tadpoles. Toxicology Letters **102-103**, 205-209.

133 Grisaru, D., Sternfeld, M., Eldor, A., Glick, D. and Soreq, H. (1999). Structural roles of acetylcholinesterase variants in biology and pathology. Eur. J. Biochem. **264**, 672-686.

134. Kaufer, D., Friedman, A., Seidman, S. and Soreq, H. (1999). Anticholinesterases induce multigenic transcriptional feedback response suppressing cholinergic neurotransmission. Chemical-Biological Interactions **14**, 119-120, 349-360.

135. Salmon, A., Goren, Z., Avissar, Y., Gluzman, N. and Soreq, H. (1999). Human erythrocyte but not brain acetylcholinesterase hydrolyzes heroin to morphine. Clinical and Experimental Pharmacology and Physiology **26**, 596-600.

136. Broide, R.S., Grifman, M., Loewenstein, A., Grisaru, D., Stone, J., Patrick, J.W., Timberg, R., Shani, M. and Soreq, H. (1999). Manipulations of ACHE gene expression suggest non-catalytic involvement of acetylcholinesterase in the functioning of mammalian photoreceptors but not in retinal degeneration. Mol. Brain Res., **71**, 137-148.

137. Perry, E., Martin-Ruiz, C., Lee, M., Griffiths, M., Johnson, M., Piggott, M., Haroutunian, V., Davis, K., Gotti, C., Tzartos, S., Cohen, O., Soreq, H., Jaros, E., Perry, R. and Court, J. (2000) Nictotinic receptor subtypes in human brain ageing and dementia. Eur. J. Pharmacol. **393**, 215-222.

138. Itskovitz-Eldor, J., Karsenti, D., Schuldiner, M., Eden, A., Yanuka, O., Amit, M., Soreq, H. and Benvenisty, N. (1999). Differentiation of human ES cells into Embryoid bodies comprising the three embryonic germ layers. Molecular Medicine **6**, 88-95 (Cover)..

139. Lev-Lehman, E., Evron, T., Broide, R.S., Meshorer, E., Ariel, I., Seidman, S and Soreq, H. (2000). Synaptogenesis and myopathy under acetylcholinesterase overexpression. J. Mol. Neuroscience **14**, 93-105.

140. Shapira, M., Tur-Kaspa, I., Bosgraaf, L., Livni, N., Grant, A.D., Grisaru, D., Korner, M., Ebstein, R.P. and Soreq, H. (2000). A transcription-activating polymorphism in the *ACHE* promoter associated with acute sensitivity to anti-cholinesterases. Hum. Mol. Genetics, **9**, 1273-1281.

141. Shohami, E., Kaufer, D., Chen, Y., Seidman, S., Cohen, O., Ginzberg, D., Melamed-Book, N., Yirmiya, R. and Soreq, H. (2000). Antisense prevention of neuronal damages following head injury in mice. J. Mol. Med., **78**, 228-236.

142. Sternfeld, M., Shoham, S., Klein, O., Flores-Flores, C., Evron, T. Idelson, G.H., Kitsberg, D., Patrick, J.W. and Soreq, H. (2000). Excess "readthrough" acetylcholinesterase attenuates but the "synaptic" variant intensifies neurodeterioration correlates. Proc. Natl. Acad. Sci. USA, **97**, 8647-8652.

143. Galyam, N., Grisaru, D., Melamed-Book, N., Grifman, M., Eckstein, F., Eldor, A. and Soreq, H. (2001) Complex host cell responses to antisense suppression of ACHE gene expression. Antisense and Nucleic Acid Drug Development, **11**, 51-57.

144. Grisaru, D., Deutsch, V., Shapira, M., Pick, M., Sternfeld, M., Melamed-Book, N., Kaufer, D., Galyam, N., Lessing, B., Gait, M., Owen, D., Eldor, A. and Soreq, H. (2001). ARP, a peptide derived from the stress-associated acetylcholinesterase variant has hematopoietic growth promoting activities. Mol. Med., **7**, 93-105.

145. Shapira, M., Thompson, C., Soreq, H and Robinson, G.E. (2001). Changes in neuronal acetylcholinesterase gene expression are temporally and functionally associated with honey bee division of labor. J. Mol. Neurosc., 17, 1-12.

146. Erb, C., Troost, J., Kopf, S., Schmitt, U., Löffelholz, K., Soreq, H. and Klein, J. (2001) Compensatory mechanisms enhance hippocampal acetylcholine release in transgenic mice expressing human acetylcholinesterase. J. Neurochem., 77, 638-646.

148. Soreq, H. and Seidman, S. (2001). Acetylcholinesterase – new roles for an old actor. Nature Neuroscience Reviews 2, 294-302.

149. Mor, I., Grisaru, D., Evron, T., Richler, C., Wahrman, J., Sternfeld, M., Teitelbaum, L., Meiri, N., Yogev, L., Seidman, S. and Soreq, H. (2001). Modified testicular expression of stress-associated "readthrough" acetylcholinesterase predicts male infertility. FASEB J., 15, 2039-2041. http://www.fasebj.org/cgi/content/abstract/00-0814fjev1?ijkey=BgvQstzaSUyaM&keytype=ref&siteid=fasebj

150. Mor, T.S., Sternfeld, M., Soreq, H., Arntzen, C.J. and Mason, H.S., (2001). Expression of recombinant human acetylcholinesterase in transgenic tomato plants. Biotechnology and Bioengineering, 75, 259-266.

151. Meshorer, E., Erb, C. Gazit, R., Pavlovsky, L., Kaufer, D., Glick, D., Friedman, A., Ben-Arie, N. and Soreq, H. (2002). Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. Science, 295, 508-512.

152. Perry, C., Eldor, A. and Soreq, H. (2002). Runx1/AML1 in Leukemia: Disrupted Association with Diverse Protein Partners. Leukemia Research, 3:221-8.

153. Grant, A.D., Shapira, M. and Soreq, H. (2001). Genomic dissection reveals locus response to stress for mammalian acetylcholinestersase. Cell Mol. Neurobiol., 21, 783-97.

154. Tomkins, O., Kaufer, D., Shelef, I., Hertzanu, Y., Richenthal, E., and Soreq, H., Friedman, A. (2001). Frequent blood-brain barrier disruption in the cerebral cortex. Cell. Mol.. Neurobiol., 21, 675-91.

155. Flores-Flores, C., Nissim, A., Shochat, S. and Soreq, H. (2002) Phage display selection and characterization of myc-tagged humanized monoclonal antibodies to synaptic acetylcholinesterase. J Neural Transm Suppl., 62, 165-179.

156. Shapira, M., Grant, A., Korner, M., and Soreq, H. (2002) Human acetylcholinesterase gene expression is controlled by an exceptionally long stress-responding promoter. I. J. Psychiatry and Behavioral Sciences, in press.

157. Cohen, O., Erb, C., Ginzberg, D., Pollak, Y., Shohami, S., Seidman, S., Soreq, H., and Yirmiya, R. (2002). Overexpression of "readthrough" acetylcholinesterase is associated with antisense suppressible behavioral impairments. Mol. Psych. 7, 874–885.

158. Svedberg, M.M., Svensson, A.-L., Johnson, M., Lee, M., Cohen, O., Court, J., Soreq, H., Perry, E. and Nordberg, A. (2002). Upregulation of neuronal nicotinic receptor subunits $\alpha 4$, $\beta 2$ and $\alpha 7$ in transgenic mice overexpressing human acetylcholinesterase. J. Mol. Neurosc., 18, 211-222.

159. Deutsch, V.R., Pick, M., Grisaru, D., Perry, C., Hemo, Y., Golan-Hadari, D., Grant, A., Eldor, A. and Soreq, H. (2002). The acetylcholinesterase variant AChE-R is expressed in human CD34$^+$ hematopoietic progenitors, and its C-terminal peptide ARP promotes their proliferation. Experimental Haematology, 30, 1153-1161.

160. Darreh-Shori, T., Almkvist, O., Guan, Z.Z., Garlind, A. Strandberg, B., Svensson, A.-L., Soreq, H., Hellström–Lindahl, E. and Nordberg A. (2002). Sustained cholinesterase inhibition in AD patients receiving rivastigmine for 12 months. Neurology, 59, 563-572.

161. Perry, C., Sklan, E., Birikh, K., Shapira, M., Trejo, L., Eldor, A. and Soreq, H. (2002). Complex regulation of acetylcholinesterase gene expression in human brain tumors. Oncogene, 21, 8428-8441.

162. Brenner, T. Hamra, Y., Evron, T., Boneva, N., Seidman, S. and Soreq, H. (2003). The role of readthrough acetylcholinesterase in the pathophysiology of myasthenia gravis. FASEB J., 17, 214-222.

163. Stoilov, P., Meshorer, E., Gencheva, M., Glick, D., Soreq., H. and Stamm, S. (2003). Defects in pre-mRNA processing as causes of and predisposition to diseases. DNA and Cell Biol., 18, 803-818.

164. Farchi, N., Soreq, H. and Hochner, B. (2003). Chronic acetylcholinesterase overexpression induces multileveled aberrations in neuromuscular physiology. J. Physiol., 546.1, 165-173.

165. Rees, T., Hammond, P. I., Soreq, H., Younkin, S.and Brimijoin. S. (2003) Acetylcholinesterase promotes beta-amyloid plaques in cerebral cortex. Neurobiology of Aging, 5810, 1-11.

166. Birikh, K., Sklan, E., Shoham, S. and Soreq, H. (2003). Interaction of "Readthrough" acetylcholinesterase with RACK1 and PKCβII correlates with intensified fear induced conflict behavior. PNAS, 100, 283-288.

167. Cohen, O., Reichenberg, A., Perry, C., Ginzberg, D., Pollmächer, T., Soreq, H. and Yirmiya, R. (2003). Endotoxin-induced changes in human working and declarative memory are associated with C - terminal cleavage of plasma "readthrough" acetylcholinesterase. J. Molec. Neurosci., 21, 199-212.

168. Nijholt, I., Farchi, N., Kye, M.-J., Sklan, E., Shoham, S., Verbeurre, B., Owen D., Hochner, B., Spiess, J., Soreq, H. and Blank., T. (2004). Alternative splicing modulation of hippocampal long-term potentiation and fear memory. Mol. Psych., 9, 174-183.

169. Darreh-Shori, T., Hellström-Lindahl, E., Flores-Flores, C, Guan, Z., Soreq, H. And Nordberg, A. (2004). Long-lasting acetylcholinesterase splice variations in anticholinesterase-treated Alzheimer's disease patients. J. Neurochem., 88:1102-1113.

170. Perry, C. Sklan E.H. and Soreq, H. (2004). CREB regulates AChE-R-induced proliferation of human glioblastoma cells. Neoplasia, 6, 279-286.

171. Sklan, E.H, Lowenthal, A., Korner, M., Ritov, Y., Rankinen, T., Bouchard, C., Leon, A.S., Rao, D.C., Wilmore, J.H., Skinner, J.S. and Soreq, H. (2004). Acetylcholinesterase/paraoxonase genotype and expression predict anxiety scores in Health, Risk Factors, Exercise Training, and Genetics study. PNAS, 101, 5512-5517.

172. Meshorer, E., Toiber, D., Zurel, D., Dori, A., Sahly, I., Cagnano, E., Grisaru, D., Tronche, F. and Soreq, H. (2004) Combinatorial Complexity of 5' Alternative ACHE Transcripts and Protein Products. J. Biol. Chem., 279, 29740-29751.

173. Dori, A., Cohen J., Silverman, W.F., Pollack, Y. and Soreq H. (2004). Functional Manipulations of Acetylcholinesterase Splice Variants Highlight Alternative Splicing Contributions to Murine Neocortical Development. Cerebral Cortex, in press.

174. Fletcher, S.P., Geyer, B.C. Smith, A., Evron, T., Joshi, L., Soreq, H. and Mor, T.S (2004). Tissue Distribution of Cholinesterases and Anticholinesterases in Tomato Plants. Plant Molecular Biology, 55, 33-44.

175. Perry, C. and Soreq, H. (2004). Organophosphate risk of leukemogenesis. Leuk Res. 28, 905-6.

176. Dong, H, Xiang, Y.-Y., Farchi, N., Ju, W., Wu, Y., Chen, L., Wang, Y., Hochner, B., Yang, B., Soreq, H. and Lu, W.-Y. (2004). Excess acetylcholinesterase impairs glutamatergic synaptogenesis in hippocampal neurons. J. Neurosci, 24, 8950-60.

177. Pick, M., Flores-Flores, C., Grisaru, D. Shochat, S., Deutsch, V. and Soreq, H. (2004). Blood cells-specific acetylcholinesterase splice variations under changing stimuli. Intl. J. Dev. Neurosci., 22, 523-31.

178. Ben-Shaul, Y. Bergman, H. and Soreq, H. (2004) Identifying Subtle Interrelated Changes in Functional Gene Categories using Continuous Measures of Gene. Bioinformatics, in press.

179. Stamm, S., Ben-Ari, S., Rafalska, I., Yesheng-Teng, Zhayi-Zhang, Debora Toiber, D., Thanaraj, A. and Soreq, H. (2004). Function of alternative splicing. GENE, in press.

180. Bryk, B., BenMoyal-Segal, L., Podoly, E., Livnah, O., Eisenkraft, A., Luria, S., Cohen, A., Yehezkelli, Y., Hourvitz, A. and Soreq, H. (2004). Inherited and acquired interactions between ACHE and PON1 polymorphisms modulate plasma acetylcholinesterase and paraoxonase activities. J. Neurochem., in press.

181. Pollak, Y., Ben-Menachem, O, Gilboa A., Ben-Hur, T., Hermona Soreq, H. and Yirmiya, R. (2004). Acetylcholinesterase modulates interleukin-1β production in the hippocampus and blood. Annals . Neurol., in press.

REVIEWS AND BOOK CHAPTERS

1. Littauer, U. Z., Salomon, R., Soreq, H., Fleischer, G. and Sela, I. (1975) Characterization of the aminoacylated region at the 3'-terminus of TMV RNA. In: Organization and Expression of the Viral Genome. Molecular Interaction in Genetic Translation. Proc. 10th FEBS Meeting, Paris, Amsterdam, Elsevier 39, 133-139.

2. Marbaix, G., Huez, G. and Soreq, H. (1977) A role for the polyadenylate segment in the stability of eukaryotic messenger RNAs. Trends in Biochemical Sciences 11, 106-107.

3. Marbaix, G., Huez, G., Soreq, H., Gallwitz, D., Weinberg, E., Devos, R., Hubert, E. and Cleuter, Y. (1979) Role of the polyadenylate segment in the stability of eukaryotic messenger RNAs. In: Gene Functions - 12th FEBS Meeting, Dresden, 1978 (S. Rosenthal, ed.). Pergamon Press, Oxford, pp. 427-432.

4. Littauer, U. Z., Soreq, H. and Cornelis, P. (1980) Polynucleotide phosphorylase as a probe for the regulatory function of the 3'-OH region of mRNA and viral RNA in translation. In: Enzyme Regulation and Mechanism of Action. (P. Mildner and B. Reis, eds.). Pergamon Press, New York, pp. 233-243.

5. Soreq, H. and Miskin, R. (1980) Screening of the protease plasminogen activator in the developing mouse brain. In: Neurotransmitters and Their Receptors. (U. Z. Littauer et al., eds.). Wiley, London, p. 559-563.

6. Littauer, U. Z. and Soreq, H. (1982) Regulatory function(s) of 3'-end sequences in translatable RNA. Prog. Nuc. A. Res. 27 (Waldo E. Cohen, ed.). Academic Press, New York, pp. 53-83.

7. Littauer, U. Z. and Soreq, H. (1982) Polynucleotide Phosphorylase. In: The Enzymes, Vol. XV, pp. 517-553.

8. Soreq, H., Parvari, R. and Silman, I. (1982) Biosynthesis of acetylcholinesterase in rat brain and Torpedo electric organ is directed by scarce mRNA speices. Prog. in Br. Res. 58, 107-115.

9. Soreq, H. (1984) Bioassays of oocyte-produced brain enzymes. In: Molecular Biology Approach to the Neurosciences. IBRO Handbook Series, methods in the neurosciences. Vol. 7. John Wiley and Sons (London, New York). Soreq, H. (ed.) pp. 187-194.

10. Burmeister, M. and Soreq, H. (1984) Production and analysis of radioactivity labeled translation products in microinjected oocytes.
IBRO Handbook Series, methods in the neurosciences. Vol. 7. John Wiley and Sons (London, New York). Soreq, H. (ed.) pp. 195-211.
11. Parvari, R., Silman, I. and Soreq, H. (1983) Ontogenetic and agranulation-induced alterations in cholinesterases and in cholinesterase mRNA in the rodent cerebellum. In: Cholinesterases - Fundamental and Applied aspects. M. Brzin, ed. W. de Groyter, Berlin, New York, pp.219-228.
12. Miskin, R. and Soreq, H. (1983) Human and rat mRNAs are translated into Urokinase-type and tissue plasminogen activators in microinjected Xenopus oocytes. In: Progress in Fibrionolysis, Vol. 6, F. Davidson, F. Brachman, C. A. Bouvier and E. K. O. Kruith, eds. Churchill Livingstone, Edinburgh, pp. 257-269.
13. Soreq, H. (1984) Post-transciptional regulation of ontogenetically modulated proteins in the nervous system. In: The Role of cell interactions in early neurogenesis. A. M. Dupart, A. C. Kato and M. Weber, eds., Plenum Press, pp. 131-138.
14. Soreq, H., Zevin-Sonkin, D., Avni, A., Hall, L. H. C. and Spierer, P. (1984) Human cholinesterase gene is homologous to the Ace locus in Drosophila. In: Dev. Neurosci: Physiological, Pharmacological and Clinical Aspects, F. Caciagli, E. Giacobini and R. Paoletti, eds. Elsevier Science Publishers B. V., pp. 95-99.
15. Soreq, H. (1985) Biosynthesis of biologically active proteins in mRNA-microinjected Xenopus oocytes. Critical Reviews in Biochemistry, 18, 199-238.
16. Zakut, H. and Soreq, H. (1985) Present and future approaches for the detection of successful clinical pregnancies with normal genetic profiles in in vitro fertilization. In: Future Neonate (S. Papadia, D. Dodero and A. Campana, eds.) Monduzzi Press, pp. 149-162.
17. Soreq, H., Zevin-Sonkin, D., Goldberg, O. and Prody, C. (1987) Molecular biology approach to the expression and properties of mammalian cholinesterases. In: Current Topics in Neurobiology: Molecular Neurobiology. S. Heinemann and J. Patrick, eds. Plenum Publishing Corp., pp. 191-224.
18. Soreq, H. and Zakut, H. (1986) Regulation and biosynthesis of cholinesteases in the human brain. In: New concepts in Alzheinmer's disease. Briley, M., Kato, A. C. and Weber, M., eds., Pierre Fabre Monograph Series, Vol. I, pp. 140-156.
19. Soreq, H. and Gnatt, A. (1987) Molecular Biological Search for human genes encoding cholinesterases. Molecular Neurobiology 1, 47-80.
20. Soreq, H., Livneh, A. and Zakut, H. (1987) The role of human antibodies towards acetylcholinesterase in neuromuscular dysfunction. Ann. N.Y. Acad. Sci. 505, 769-772.
21. Gnatt, A. and Soreq, H. (1987) Molecular cloning of human cholinesterase genes: Potential applications in neurotoxicology. In: Model systems in Neurotoxicology: Alternative approaches to Animal Testing. Eds. A. Shahar and A. M.. Goldberg, Alan R Liss, Inc. New York, pp. 111-119.
22. Soreq, H. and Prody, C. (1989) Sequence similarities between human acetylcholinesterase and related proteins: Putative implications for therapy of anticholinesterase intoxication. In: Computer-assisted modeling of receptor-ligand interactions, theoretical aspects and applications to drug design. (Golombek, A. and Rein, R., eds.), Alan R. Liss, New York, pp. 347-359.
23. Soreq, H. and Zakut, H. (1990) Amplification of butyrylcholinesterase and acetylcholinesterase genes in normal and tumor tissues: Putative relationship to organophosphorous poisoning. Pharmaceutical Research, 7, 1-7.
24. Seidman, S. and Soreq, H. (1990) Co-injection of Xenopus oocytes with cDNA-produced and native mRNAs: a Molecular Biological approach to the tissue specific processing of human cholinesterases. Int. Rev. in Neurobiology, 32, 107-139.
25. Ehrlich, G., Soreq, H. and Zakut, H. (1990) Gene amplification in primary human tumors. HaRefuah, 118, 579-586 (Hebrew).
26. Soreq, H. (1990) Elements of Molecular Neurobiology (an invited Book Review) Trends in Neurosci., 13, 351-352.
27. Ayalon, A., Zakut, H., Prody, C.A. and Soreq, H. (1990) Preferential transcription of acetylcholinesterase over butyrylcholinesterase mRNAs in fetal human cholinergic neurons. In: Gene Expression in the nervous system. (A.M. Giuffrida-Stella, Ed.) Alan R. Liss, New York pp. 191-203.
28. Soreq, H. and Zakut, H. (1990) Expression and in vivo amplification of the human cholinesterase genes. Progress in Brain Research. Vol. 84 S.-M. Aquilonius and P.-G. Gillberg, Eds. Elsevier Science Publishers B.V. (Amsterdam) pp. 51-61.
29. Soreq, H. (1990) Cholinesterases in cellular and molecular neurobiology. Cell. Mol. Neurobiol. 11, 3-5.
30. Dreyfus, P.A., Pincon-Raymond, M., Zakut, H., Seidman, S. and Soreq, H. (1991) Search for the molecular origins of butyrylcholinesterase polymorphism by cDNA screening, deletion mutagenesis and Xenopus oocytes co-injections. In: Proceedings of the Third International Meeting on Cholinesterases. ACS books, Washington, D.C., J. Massoulie, F. Bacou, E. Barnard, A. Chatonnet, B.P. Doctor and D.M. Quinn, eds. pp. 162-167.
31. Ben Aziz, R., Gnatt, A., Prody, C.A., Lev-Lehman, E., Neville, L., Seidman, S., Ginzberg, D., Soreq, H., Lapidot-Lifson, Y. and Zakut, H. (1991) Differential codon usage and distinct surface probabilities in human acetylcholinesterase and butyrylcholinesterase. In: Proceedings of the Third International Meeting on

Cholinesterases. ACS books, Washington, D.C. J. Massoulie, F. Bacou, E. Barnard, A. Chatonnet, B.P. Doctor and D.M. Quinn, eds. pp. 172-178.

32. Neville, L.F., Gnatt, A., Seidman, S., Loewenstein, Y., Padan, R. and Soreq, H. (1991) Modified ligand-binding properties of butyrylcholinesterase muteins produced in microinjected Xenopus oocytes. In: Proceedings of the Third International Meeting on Cholinesterases. ACS books, Washington, D.C. J. Massoulie, F. Bacou, E. Barnard, A. Chatonnet, B.P. Doctor and D.M. Quinn, eds. pp. 197-198.
33. Soreq, H., Neville, L., Gnatt, A., Ben-Aziz, R., Lapidot-Lifson, Y., Ehrlich, G., Seidman, S., Lev-Lehman, E., Beeri, R., Ginzberg, D. and Zakut, H. (1991) Structure-function relationships, in vivo mutability and gene amplification in human cholinesterases, targets for organophosphorous poisons. In: Biotechnology: Bridging Research and Applications, Kamely D, Chakrabarty A.M., Kornguth S.E., eds. Kluwer Academic Publishers, Boston pp. 153-166.
34. Soreq, H., Loewenstein, Y., Neville, L.F., Seidman, S., Ben-Aziz, R., Ehrlich, G., Gnatt, A. and Ginzberg, D. (1991) Structure-function relationship studies in human cholinesterases as an approach for evaluating potential pharmacotherapeutic and/or toxicity effects of cholinergic drugs. In: Cholinergic Basis for Alzheimer Therapy. R.E. Becker and E. Giacobini, Eds. Birkhauser, Boston. pp. 46-52.
35. Soreq, H., Lapidot-Lifson, Y. and Zakut, H. (1991) Role for cholinesterases in Tumorigenesis? Cancer Cells, 3, 511-516.
36. Soreq, H., Ehrlich, G., Gnatt, A., Neville, L., Ben-Aziz-Aloya, R., Seidman, S., Ginzberg, D. Lapidot-Lifson, Y. and Zakut, H. (1992) Amplification and mutagenesis of the acetylcholinesterase and butyrlcholinesterase genes in primary human tumors. In: Gene Amplification in Mammalian Cells. R.E. Kellems, ed. Marcel Dekker, Inc., New York pp. 417-428.
37. Soreq, H. and Seidman, S. (1992) Xenopus oocyte microinjection: from genes to proteins. Methods Enzymol. 207, 225-265.
38. Beeri, R., Gnatt, A. Lapidot-Lifson, Y., Ginzberg, D., Shani, M., Zakut, H. and Soreq, H. (1992) Testicular Gene Amplification and impaired BCHE transcription induced in transgenic mice by the human BCHE coding sequence. Multidisciplinary approaches to cholinesterase functions. A. Shaefferman and B. Velan, Eds. Plenum Press, New York pp. 91-94.
39. Gnatt, A., Loewenstein, Y., Ben Aziz - Aloya, R. and Soreq, H. (1992) Molecular dissection of functional domains in human cholinesterases expressed in microinjected Xenopus oocytes. Multidisciplinary approaches to cholinesterase functions. A. Shaefferman and B. Velan, Eds. Plenum Press, New York pp. 157-164.
40. Zakut, H., Lapidot-Lifson, Y., Patinkin, D., Ginzberg, D., Ehrlich, G., Eckstein, F. and Soreq, H. (1992) Clinical implications of cholinesterase aberrations in syndromes of hemopoietic cell division. In: Multidisciplinary approaches to cholinesterase functions. A. Shaefferman and B. Velan, Eds. Plenum Press, New York pp. 269-276.
41. Ben Aziz-Aloya, R., Sternfeld, M. and Soreq, H. (1993) Promoter elements and alternative splicing options in the human ACHE gene. In: CNS cholinergic Function and Dysfunction. Prog. Brain Res. 48, 147-153.
42. Soreq, H., Beeri, R., Seidman, S., Timberg, R., Loewenstein, Y., Sternfeld, M. and Andres, C. (1994) Modulating cholinergic neurotransmission through transgenic overexpression of human cholinesterases. In: Alzheimer Therapy: Therapeutic Strategies, E. Giacobini and R. Becker, Eds. Birkhauser, Boston. pp. 84-87.
43. Soreq, H., Loewenstein, Y., Glick, D. and Zakut, H. (1994) Is aluminum salt-activated acetylcholinesterase subject to the genetic predisposition that protects the enzyme from inhibitors? Neuroreport 5, 14.
44. Ehrlich, G., Ginzberg, D., Schwarz, M., Loewenstein, Y., Glick, D., Kerem, B., Ben-Ari, S., Zakut, H. and Soreq, H. (1995) Population diversity of point mutations in the human ACHE and BCHE genes predicts variable responses to anti-cholinesterase drugs. In: Alzheimer's and Parkinson's Diseases, Hanin, I. et al. eds. Plenum Press, New York, pp. 661 - 667.
45. Lev-Lehman, E., El-Tamer, A., Ginzberg, D., Hanin, I. and Soreq, H. (1995) Transient alterations in the in vivo levels of cholinesterase mRNAs suggest differential adjustment to cholinotoxic stimuli. In: Alzheimer's and Parkinson's Diseases: Recent Advances, Hanin, I., Yoshida, M. and Fisher, A. Eds. Plenum Press, New York, pp. 347-352.
46. Hanin, I., Yaron, A., Ginzberg, D. and Soreq, H. (1995) The cholinotoxin AF64A differentially attenuates the in vitro transcription of human cholinesterase genes. In: Alzheimer's and Parkinson's Diseases: Recent Advances, Hanin, I., Yoshida, M. and Fisher, A. Eds. Plenum Press, New York, pp. 339-345.
47. Schwarz, M., Glick, D., Loewenstein, Y. and Soreq, H. (1995) Engineering of human cholinesterases explains and predicts diverse consequences of administration of various drugs and poisons. Pharmac. Ther. 67, 283-322.
48. Soreq, H., Lev-Lehman, E., Patinkin, D., Grifman, M., Ehrlich, G., Ginzberg, D., Eckstein, F. and Zakut, H. (1995) Antisense oligonucleotides suppressing expression of cholinesterase genes modulate hematopoiesis in /vivo and ex-vivo. In: Enzymes of the Cholinesterases Family. Quinn, D.M., Balasubramanian, A.S., Doctor B.P. and Taylor, P. Eds. Plenum Press, New York, pp 1-6.
49. Andres, C., Beeri, R., Huberman, T., Shani, M. and Soreq, H. (1996) Cholinergic drug resistance and impaired spatial learning in transgenic mice overexpressing human brain acetylcholinesterase. Progress in Brain Res. 109, 265-272.

50. Kaufer-Nachum, D., Friedman, A., Sternfeld, M., Seidman, S., Beeri, R., Andres, C. and Soreq, H. (1996). Central and peripheral consequences of cholinergic imbalance in Alzheimer's disease. In: Alzheimer Disease: From Molecular Biology to Therapy. Giacobini, E. and Becker, R. eds. Birkhauser, Boston, pp. 153-158.

51. Soreq, H. (1996). Synaptic transmission - cellular and molecular basis. A book review on H. Zimmermann's Monography (Thieme, Stuttgart, 1994). J. Neurochem. 66, 2626.

52. Grifman, M., Lev-Lehman, E., Ginzberg, D., Eckstein, F., Zakut, H. and Soreq, H. (1997). Potential antisense oligonucleotide therapies for neurodegenerative diseases. In: Concepts in Gene Therapy, M. Strauss and J.A. Barranger, Eds. Walter de Gruyter & Co., Berlin. pp. 141-167.

53. Sternfeld, M., Seidman, S., Beeri, R. and Soreq, H. (1997) Catalytic and non-catalytic acetylcholinesterase implied from Transgenic ACHE Expression in vertebrates. In: Neurotransmitter Release and Uptake, NATO ASI Series. Vol. H100, S. Pogun, Ed., Springer, Berlin, pp 301-308.

54. Sternfeld, M., Sela, K., Seidman, S., Timberg, R. and Soreq, H. (1997) Molecular dissection of protein domains directing the tissue targetting of Acetylcholinesterase isoforms. In: Neurotransmitter Release and Uptake, NATO ASI Series. Vol. H100, S. Pogun, Ed., Springer, Berlin, pp 309-316.

55. Soreq, H. and Glick, D. (1997). Transgenic manipulations of cholinergic functions in vertebrates. In: Proceedings of the Second Chemical and Biological Medical Treatment Symposium, NC-Laboratory of Spiez, Spiez, Switzerland, 7-12 July 1966, B. Price, Ed. Applied Science and Analysis, Portland, Maine, USA, 152-154.

56. Soreq, H., Kaufer, D., Beeri, R. and Friedman A. (1997) Short and long term manipulations of cholinergic functions under stress conditions and in transgenic animals. Chemistry 34 (Hebrew):102-103.

57. Grifman, M. Ginzberg, D. and Soreq, H. (1998) Antisense oligodeoxynucleotide-dependent suppression of acetylcholinesterase expression reduces process extension from primary mammalian neurons. In: Progress in Alzheimer's and Parkinson's Diseases: Recent Advances. Fisher, A., Hanin, I. and Yoshida, M. Eds. Plenum Press, New York and London. pp. 557-562.

58. Soreq, H. and Seidman, S. (1998) The non-catalytic role and complex management of acetylcholinesterase in the mammalian brain call for RNA-based therapies. In: Progress in Alzheimer's and Parkinson's Diseases: Recent Advances. Fisher, A., Hanin, I. and Yoshida, M. Eds. Plenum Press, New York and London. pp. 551-556.

59. Soreq, H. (1998). Cholinergic Mechanisms. In: ID Drugs 1, 787-793.

60. Soreq, H. (1998) Genetic manipulations of catalytic and non-catalytic functions of human cholinesterases. In: tructure and Function of Cholinesterases and Related Proteins, Quinn, D.M., Doctor, B.P. and Taylor, P. Eds. Plenum Press, New York. pp 35-39.

61. Kaufer, D., Friedman, A. and Soreq, H. (1999). The vicious circle of stress and anticholinesterase exposure. The Neuroscientist 5, 173-183.

62. Shapira, M., Korner, M., Bosgraaf, L., Tur-Kaspa, I. and Soreq, H. (1999). The human ACHE locus includes a polymorphic enhancer domain 17 kb upstream from the transcription start site. In: Cholinesterases and Related Proteins, Quinn, D.M., Doctor, B.P. and Taylor, P. Eds. Plenum Press, New York. pp 111.

63. Seidman, S., Cohen, O., Ginzberg, D., Eichengreen, E., Pollak, Y., Yirmiya, R. and Soreq, H. (1999). Multilevel approaches to AChE-induced impairments in learning and memory. Seidman, S., Cohen, O., Gizberg, D., Eichengreen, E., Pollak, Y., Yirmiya, R. and Soreq, H. (1999). In: Cholinesterases and Related Proteins, Quinn, D.M., Doctor, B.P. and Taylor, P. Eds. Plenum Press, New York. pp 183-184.

64. Seidman, S., Eckstein, F., Grifman, M., and Soreq, H. (1999). Antisense technologies have a future fighting Neurodegenerative diseases. Antisense Research and Nucleic Acids Drug Development 9, 333-340.

65. Soreq, H. (1999). The Molecular biology of post-traumatic stress and antisense technology. Geriatric Medicine 37, 1323-1328 [Japanese].

66. Glick, D. and Soreq, H. (1999). Brain research at the end of the millenium: New Horizons. ID Drugs 2, 983-987.

67. Kaufer, D. and Soreq, H. (1999) Tracking cholinergic pathways from psychological and chemical stressors to variable neurodegeneration paradigms. Current Opinion in Neurology 12, 739-743.

68. Sternfeld, M. and Soreq, H., (2000) Acetylcholinesterase variants in mammalian stress: catalytic short-term and structural long-term roles. In : Molecular Genetics of Mental Disorders, M. Briley, Ed Martin Dunitz London. pp. 133-154.

69. Soreq, H., Kaufer, D. and Friedman, A. (2000). The molecular biology of post-traumatic disorder. Harefuah, 138, 864-870 (Hebrew).

70. Soreq, H. (2000). Psychological and chemical stressors and the Gulf War syndrome. Encyclopedia of Stress. Academic Press, San Diego pp. 302-312.

71. Soreq, H. and Seidman, S. (2000). Antisense approach to isoform – specific blockade of acetylcholinesterase. In: Antisense Drug Technology: Principles, Strategies and Applications. S. T. Crooke, Ed. Marcel Dekker, Inc., Carlsbad, CA. pp. 565-586.

72. Soreq, H. and Seidman, S. (2000). Antisense approach to anticholinesterase therapeutics. Israel Med. Assoc. J.,2, 81-85.

73. Shapira, M., Grant, A., Korner, M., and Soreq, H. (2000) Genomic and transcriptional characterization of the human ACHE locus indicates complex involvement with acquired and inherited diseases. Israel Medical Assoc. J., 2, 470-473

74. Perry, C. and Soreq, H. (2001). The Leukemic effect of anticholinesterases. Leukemia Research, 25, 1027-1028.

75. Soreq, H. and Glick, D. (2001). Readthrough acetylcholinesterase is an antisense-suppressible stress-responding element. In: Conformational Diseases – a compendium. The Bialik Institute, Jerusalem. E. Katzir, B. Solomon and A. Taraboulos, Eds. pp. 226-231.

76. Shapira, M., Glick, D., Gilbert, J.P., and Soreq, H. (2001). Autism, stress and chromosome 7 genes. In: The Research Basis of Autism Intervention. Kluwer Academic/Plenum Publishers, NY. E. Schloper, L. Marcus, C. Shulman and N. Yirmiya, Eds. pp. 103-113.

77. Lev-Lehman, E., Seidman, S., Ginzberg, D. and Soreq, H. (2002) Antisense oligomers in vitro and in vivo. In press in: Antisense Therapeutics, A. Cohen and S. Smicek, Eds. Plenum Press.

78. Glick, D., Shapira, M., and Soreq, H. (2002). Molecular neurotoxicology implications of acetylcholinesterase inhibition. In: Site-Selective Neurotoxicology, D. S. Lester, W. Slikker, Jr. and P. Lazarovici, Eds. Plenum Press, New York, pp. 116-133.

79. Soreq, H. and Glick, D. (2002). Novel roles for cholinesterases in stress and inhibitor responses. Ezio Giacobini (Ed.) In: Cholinesterases and cholinesterase inhibitors: basic, preclinical and clinical aspects. Martin Dunitz Ltd. pp. 47-61.

80. Soreq, H., Kaufer, D., Friedman, A. and Glick, D. (2002) Blood-Brain Barrier modulations and low-level exposure to Xenobiotics. In: "Chemical Warfare Agents: Low Level Toxicity". S. M. Somani and J. A. Romano, Eds. CRC Press, Boca Raton, FL pp. 121-144.

81. Soreq, H. and Glick, D. (2002) Acetylcholinesterase. In: The Encyclopedia of Molecular Medicine.Thomas E. Creighton, Ed. Wiley pp. 14-16.

82. Soreq, H., Kaufer, D., Glick, D. and Friedman, A. (2002). The molecular biology of Blood Brain Barrier disruption under stress. In: Brain Disease: Therapeutic Strategies and Repair. O. Abramsky, D. Alastair, A. Miller and G. Said, Eds. Martin Dunitz, Publishers, pp.231-238.

83. Meshorer, E. and Soreq, H. (2002) Antisense Intervention with Cholinergic Impairment Associated with Neurodegenerative Disease. Mapping the Progress of Alzheimer's and Parkinson's Disease. Y. Mizuno, A. Fisher, I. Hanin, Eds. Kluwer Academic/Plenum Publisher. pp. 45-48.

84. Perry, C. and Soreq, H. (2002). Runx1/AML1 in leukemia: disrupted association with diverse protein partners. Leukemia Research, 26, 221-228.

85. Perry, C. and Soreq, H. (2002). Transcriptional regulation of erythropoiesis: fine tuning of combinatorial multi-domain elements. EJB, 269, 3607–3618.

86. Meshorer, E. and Soreq, H. (2002) Pre-mRNA splicing modulations in senescence. Aging Cell, 1, 10-16.

87. Cohen, O., Meshorer, E., Friedman, A., Ginzberg, D. Glick, D., Yirmiya, R. and Soreq, H. (2002) Anticholinesterase intensification and antisense suppression of molecular genetic impairments in cholinergic homeostasis. (US Army)

88. Glick, D., Ben Moyal, L. and Soreq, H. (2003) Genetic variation in butyrylcholinesterase and the physiological consequences for acetylcholinesterase function. In: Giacobini, E. (ed.) *Butyrylcholinesterase: Function and Inhibition*, Martin Dunitz , London, pp.55-67.

89. Soreq, H. Meshorer, E., Cohen, O., Yirmiya, R., Ginzberg, D. and Glick, D. (2004). The molecular neurobiology of acetylcholinesterase variants: from stressful insults to antisense intervention. In: Silman, I., Fisher, A., Anglister, L. Michaelson, D. and Soreq, H. (eds.) *Cholinergic Mechanisms*, Martin Dunitz , London, pp.119-124.

90. Evron, T. and Soreq, H. (2004) Transgenic manipulations of neuromuscular junction maintenance. . In press In: Silman, I., Fisher, A., Anglister, L. Michaelson, D. and Soreq, H. (eds.) *Cholinergic Mechanisms*, Martin Dunitz , London, pp. 567-569.

91. Sklan, E.H., Birikh, K. and Soreq H. (2004) Two hybrid approach to the intracellular function(s) of readthrough acetylcholinesterase. In press in: Silman, I., Fisher, A., Anglister, L. Michaelson, D. and Soreq, H. (eds.) *Cholinergic Mechanisms*, Martin Dunitz , London, pp. 707-709.

92. Shoham, S., Kovalev, E., Sklan, E. and Soreq, H. (2004). Readthrough acetylcholinesterase and cholinergic transmission. In press in: Silman, I., Fisher, A., Anglister, L. Michaelson, D. and Soreq, H. (eds.) *Cholinergic Mechanisms*, Martin Dunitz , London, pp. 697-700.

93. Mor, T.S. and Soreq, H. (2003). Human cholinesterases from plants for detoxification. In: *Encyclopedia of Plant and Crop Science*, Goodman R.M. (ed); (Joshi L.(section ed.), Marcel Dekker, New York. pp. 564-567.

94. Glick, D. and Soreq. H. (2003). Ethics, Public Policy and Behavioral Genetics. *IMAJ* 5: February: 83-86.

95. Ben-Shaul, Y., Bergman, H. and Soreq, H. (2003) AChE and Parkinson's disease. In press in: Alzheimer's and Parkinson's Diseases, Hanin, I. et al. eds. Plenum Press, New York.

96. Hermona Soreq, Raz Yirmiya, Osnat Cohen, David Glick. (2003). Acetylcholinestease as a window onto stress responses. Handbook on Stress, Immunology and Behaviour. Elsevier science, Amsterdam. In press.

97. Pick, M., Flores-Flores, C. and Soreq H. (2004). From brain to blood: Alternative splicing evidence for the cholinergic basis of mammalian stress responses. In Pacak K., Aguilera G., Sabban E.L., Kvetnansky R. (eds.) *Stress: Neuroendocrine and Generic Approaches*, Vol. 1018 pp. 85-98, Ann. NY Acad. Sci., N.Y. Annals Review.Online, www.annalsnyas.org.

98. Meshorer, E. and Soreq, H. (2004). Pre-mRNA modulators in stress and aging. In press in: Handbook of Neurochemistry and Molecular Neurobiology, Volume No. 14. Developmental and Aging changes Handbook of Neurochemistry and Molecular Neurology 3rd Edition Eds. J. Regino Perez-Polo and Steffen Rossner. New York: Plenum, in press

99. Soreq, H. and Meshorer, E. (2004). Differential modulations in neuronal gene expression in aging and stress. New third edition of the Handbook of Neurochemistry and Molecular Neurobiology, Volume No. 14, J. Regino Perez-Polo and Steffen Rossner, Eds. in press.

100. Sklan E.H., Shoham S. and Soreq H. (2004) Interaction of "Readthrough" Acetylcholinesterase with the PKC Scaffold Protein Rack1 Facilitates Antisense-Suppressible Contextual Fear Responses. In: Inestrosa, N.C. and Campos, E.O. (eds.), *Cholinesterases in the Second Millennium: Molecular and Pathological Aspects* P. Univ. Católica de Chile, Santiago, Chile, pp 65-74.

101. Soreq, H. (2004). Caught between the need to publish and the requirement to conceal. In press in: Shefler, G. (ed.) *Ethics in Research*, Magnes Press, Jerusalem (Hebrew).

EXHIBIT B

Acetylcholinestease as a window onto stress responses

Hermona Soreq, Raz Yirmiya, Osnat Cohen, David Glick

Abstract

Some years ago we had noted that both stress and inhibition of acetylcholinesterase (AChE) result in some common long-lasting symptoms. We then found that both stress and inhibition of AChE cause an increase in genetic expression of AChE that is also associated with a shift in its pre-mRNA splicing pattern. Of the several variants of AChE that arise due to alternative splicing, it is specifically the usually rare, soluble AChE-R variant that is up-regulated. Transgenic mice that over-express AChE also show many of the same symptoms as stress: erratic behavior following circadian light/dark shift, progressive muscle fatigue and degeneration of neuromuscular junctions, progressive failure of learning and memory, and development of neuropathologies. Stress-associated characteristics can be ameliorated by treatment with antisense agents that induce selective destruction of AChE-R, which provides further support for this variant having a role in the etiology of stress responses.

Introduction

A striking similarity exists between reported responses to anti-cholinesterase exposure and stress (Fullerton and Ursano, 1990; Somani et al., 2000). Furthermore, anti-cholinesterases and stress both induce expression of the acetylcholinesterase gene, *ACHE* (Kaufer et al., 1998; Kaufer et al., 1999). The realization of this similarity has drawn us into stress studies from backgrounds in molecular biology and neuroimmunology, and the melding of our molecular biological and behavioral approaches has contributed, we feel, to a deeper appreciation of cholinergic neurotransmission, and of acetylcholinesterase (AChE) in particular, in stress responses. This chapter, therefore, presents the cellular and behavioral long-term stress responses as molecular and biochemical processes that are amenable to further study, and, we hope, to therapeutic intervention.

Acetylcholinesterase and stress responses

Cholinergic responses to stress

It is well accepted that stress in mammals is rapidly followed by a pronounced activation of central cholinergic pathways that is correlated with transiently enhanced release of acetylcholine (ACh) (Dazzi et al., 1995; Imperato et al., 1991). Several lines of evidence suggest that this initial event contributes to the behavioral consequences of stress. For example, scopolamine affects rodent responses to foot-shock stress (Kaneto, 1997). and long-term psychological disturbances, strikingly reminiscent of those that characterize post-traumatic stress disorder (PTSD) are associated with both acute (Burchfiel and Duffy, 1982; Rosenstock et al., 1991) and chronic (Li et al., 2000; Stephens et al., 1995) exposure to inhibitors of AChE. In laboratory animals both stress and inhibition of AChE indirectly cause elevation of AChE mRNA and protein levels in the brain, while suppressing levels of mRNA that encode the ACh-synthesizing enzyme choline acetyltransferase (ChAT) (Friedman et al., 1996; Kaufer et al., 1998). These observations indicate that a decrease in ACh, due to increased hydrolysis, is re-enforced by a reduced ACh-synthesizing potential in cholinergic pathways. These complementary modulations of cholinergic gene expression ensure that transient acute cholinergic hyperactivation is followed by a persistent, bimodal suppression of cholinergic neurotransmission in the mammalian brain due to

new AChE synthesis. These clues have led us to the initiation of a systematic investigation of the role of AChE in stress responses in several experimental paradigms, including the human hematopoietic system (Grisaru et al., 2001), mouse hippocampal slices (Friedman et al., 1998) myasthenic rat muscle (Brenner et al., in press) and testicular tubules. (Mor et al., 2001). In all of these systems we found that various stressors induce expression of AChE-R mRNA, one of the 3'-AChE mRNA variants that result from alternative splicing of the transcript of the AChE gene, *ACHE* (Grisaru et al., 1999b; Soreq and Glick, 2000). These findings highlight the role of this normally rare variant of AChE in mammalian stress. The observations, in four major organ systems, suggest a central role for cholinergic pathways in mediating long-term mammalian stress responses. The scope of these observations could have been expanded to include panic disorders, which have an important cholinergic component (Battaglia, 2002), and depression (Lupien et al., 1999), but this would take us beyond the mandate of this chapter.

3'-Splicing variants

One source of the variation of AChE is 3'-alternative mRNA splicing (Fig. 1), which confers different C-terminal sequences on a 543-residue core protein.

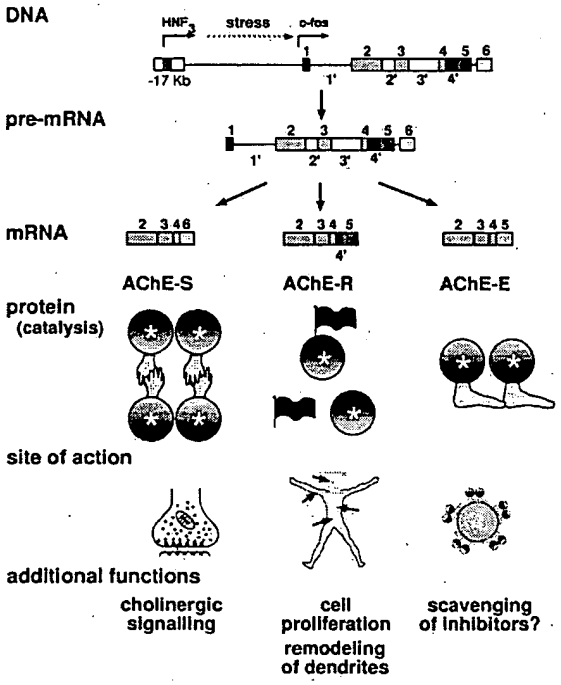


Fig. 1. The molecular biology of human AChE.

A single human *ACHE* gene gives rise to a wide variety of protein variants. The 543-residue core of human AChE is encoded by 3 exons, 2, 3 and 4 and by itself is catalytically competent; 3'-alternative splicing of the pre-mRNA result in additional C-terminal sequences of the S (synaptic), R (readthrough) and E (erythrocytic) variants. Human AChE-S terminates with 40 residues (DTLDEAERQWKAEFHRWSSYMVHWKNQFDHYSKQDRCSDL); AChE-E with

14 residues (ASEAPSTCPGFTHG); and AChE-R with 26 residues (GMQGPAGSGWEEGSGS PPGVTPLFSP). The translation start codon is in exon 2, which encodes a leader sequence that does not appear in any known mature protein. In addition to a proximal promoter next to exon 1 (Atanasova et al., 1999; Camp and Taylor, 1998; Chan et al., 1998), a far upstream distal enhancer region is rich in potential regulatory sequences. For instance, the *ACHE* transcriptional activation by cortisol (Grisaru et al., 2001) is likely due to the distal glucocorticoid response element (Meshorer et al., 2002). A deletion mutation in this region disrupts 1 of 2 HNF3 binding sites, which consequently activates transcription (Shapira et al., 2000b). Normally, much more AChE-S than AChE-R mRNA is produced, but under stress or inhibition of AChE, alternative splicing produces much more of the AChE-R mRNA, likely under c-fos regulation (Kaufer et al., 1998). The C-terminal sequence of AChE-S enables it to form multimers that can then be joined to anchoring proteins (Ohno et al., 2000; Perrier et al., 2002) that attach them to the synaptic membrane; and AChE-E dimerizes and undergoes a transesterification reaction to replace its C-terminal sequence with a glycophosphatidylinositol group that can be embedded in the erythrocyte membrane. AChE-R, however, cannot form multimers, and when secreted, it remains soluble. Additional variation arises from post-translational changes: glycosylation, which affects turnover (Kronman et al., 2000), formation of intermolecular disulfide bridges and attachment to a phospholipid or collagen-like protein anchor to synapse membranes (as mentioned), or intracellular interaction with partner proteins (Birikh et al., in press).

Note on nomenclature.
Another nomenclature, based on physical rather than molecular biological relations, names the synaptic and erythropoietic variants according to properties of the proteins: T (tailed), and H (hydrophobic), respectively (Massoulie, 2000). The many guises in which AChE-T occurs are further termed G, for globular, or A for asymmetric and G_1, G_2 etc. for monomer, dimer, etc.

Transgenic mouse models
We first created a transgenic mouse model (TgS), in which over-expression of human synaptic AChE-S, limited to central nervous system (CNS) neurons, promotes a late-onset and progressive impairment in learning and memory that is associated with PTSD (Beeri et al., 1995). The cognitive defects observed in these mice were assumed to reflect the physiological state induced by an excess of AChE. The possibility that modified regulation of *ACHE* gene expression may have imposed profound disruption of both central and peripheral cholinergic systems is strengthened by our more recent findings of multileveled impairments in neuromuscular junction (NMJ) structure and function (Farchi et al., in press), which eventually led to amyotrophy in AChE-transgenic mice (Andres et al., 1997). However, there is a temporal gap between over-expression of AChE in young TgS mice and the delayed onset of neurodegenerative and cognitive processes, similar to the gap observed in human carriers of mutations that increase the risk for late-onset diseases.

That too little AChE is harmful is powerfully demonstrated by the lethality of anti-AChE nerve agents (Taylor, 1996). The fact that AChE-knockout mice can survive in a protected environment seems to indicate that to a very limited extent the homologous enzyme butyrylcholinesterase (BuChE) can take over some of the functions of AChE (Mesulam et al., 2002) but in no way invalidates this conclusion. That an excess of synaptic AChE-S is also harmful, albeit in a very different way, has been demonstrated in studies of TgS mice; an excess of the AChE-R variant

seems more benign in brain and muscle (Andres et al., 1997; Beeri et al., 1995; Sternfeld et al., 2000), and may even explain the protective nature of the splicing changes in response to stress. However, AChE-R accumulation also has adverse consequences, e.g. it impairs sperm maturation and is associated with male infertility (Mor et al., 2001).

Acetylcholinesterase and delayed-onset neurodegeneration

A consideration of the mass of data on stress responses indicates an intimate involvement of *ACHE* gene expression in both the natural response to various stresses and in the adaptation to changed environment that delays neurodegeneration. Several diseases of the central and peripheral nervous system are characterized by adult-onset, progressive deterioration. Examples include Alzheimer's disease (AD) (Selkoe, 2002), spinal muscular atrophy (Nicole et al., 2002), myasthenia gravis (Vincent and Drachman, 2002), Eaton-Lambert disease (Zenone et al., 1992) and Parkinson's disease (PD) (Bergman and Deuschl, 2002). Despite their different origins and rates of progress, similar symptoms may result from these various etiologies, e.g. AD and PD (Greenfield and Vaux, 2002). Additionally, an interesting and revealing parallel exists between the long-term effects of anti-AChEs and these delayed-onset neurodegenerative diseases (Kaufer et al., 1998; Millard and Broomfield, 1995). A simple model to explain the delayed-onset of pathology in these degenerative conditions would view the decline toward disease as a gradual accumulation of damage in a basic process that is essential for maintenance of these functions, and which results in pathology beyond a certain threshold. In such a model, built-in margins of safety protect the system, and hence the organism, for a period of time that reflects the size of the safety margin. A more complex approach to understanding late-onset disease is to postulate adaptation to the change, for example by feedback responses retrieving the impaired process and preventing perturbations in normal function during embryonic, post-natal, and young adult periods. While such adaptations can enable normal functioning in the short term, they may eventually result in a delayed pathology. In that case, the age of onset of symptoms will depend on the limits of compensation and on the functional integrity of the cellular and molecular mechanisms that regulate adaptive pathways. Yet more specifically, the data point to changes in the control over AChE's alternative splicing as the key process that enables these changes in *ACHE* expression.

One of the strategies by which Nature has greatly expanded the expression potential of the genome is by alternative splicing of pre-mRNAs. In many cases, the original transcript of the gene can generate a number of different mature mRNAs by selection of only some of the open reading frames and elimination of others. Thus subtly or substantially different variant proteins may be generated from a single gene. The process of splicing is accomplished by complex molecular motors, under the direction of a number of sometimes competing small splicing factor proteins (Stamm, 2002). The limited number of these factors, and the balance among them, would allow a concerted response to an external change by the generation of characteristic splicing variants of a potentially large number of genes. Thus, a stress, starvation for example, may initially bring about the up-regulation of a single splicing factor, which because of its involvement in splicing of a wide variety of pre-mRNAs, results in the biosynthesis of the proteins that can minimize the effects of starvation. The integration of the response is built into the participation of that one (or those few) splicing factor(s) in the synthesis by splicing of the physiologically appropriate mRNAs. Similarly, a defect, inherited or acquired, in one of these splicing factors would affect a correspondingly wide variety of such events. Although the extent

of alternative splicing as an integrating (or disintegrating) principle is still being explored, it is a fruitful source of hypotheses that may help to explain the many forms that responses to stress may take in different tissues, and the similarities among responses to various kinds of stresses. One example taken from the neurosciences is the stress-induced modification of K^+ channels (Xie and Black, 2001; Xie and McCobb, 1998), which is regulated by neuronal activity-dependent transcriptional changes in a number of splicing regulatory proteins (Daoud et al., 1999). Another example, of course, is the stress-directed splicing of AChE pre-mRNA (Meshorer et al., 2002; Soreq and Glick, 2000). It has been our burden to explore the physiological consequences of this phenomenon.

Transcriptional feedback response to stress

The functioning of the *ACHE* gene is subject to massive developmental pressures, yet retains a certain level of plasticity also in the adult. In both neocortical and hippocampal neurons, various external stimuli induce rapid, yet long-lasting activation of *ACHE* gene expression. In fact, the *ACHE* gene responds with increased transcription to psychological stress (Kaufer et al., 1998), anti-AChE intoxication (Shapira et al., 2000b), closed head injury (Shohami et al., 2000) and autoimmune impairments of neuromuscular function (Brenner et al., in press), alike. It is presumed that psychological or physical stress induces cholinergic excitation via release of ACh. Elevated cortisol and the consequent feedback over-expression of AChE then act to dampen excessive neurotransmission toward its normal level (Kaufer et al., 1998). This is important both for cholinergic neurotransmission and for other neurotransmission circuits modulated by ACh, for example hippocampal glutamatergic activity (Gray et al., 1996; Meshorer et al., 2002), and dopaminergic circuits in the substantia nigra (Llinas and Greenfield, 1987). That excess AChE can also protect the organism from the toxicity of anti-AChEs, has been demonstrated in laboratory animals (Ashani et al., 1991; Doctor et al., 1993; Raveh et al., 1989; Wolfe et al., 1992).

Transcriptional activation is common to many genetically determined responses to pharmaceuticals, e.g. by cytochrome P-450 proteins. (Evans and Relling, 1999). However, in addition to transcriptional activation, AChE mRNA transcripts in nerve, muscle and blood cells are subject to calcineurin-controlled differentiation-induced stabilization (Chan et al., 1998; Luo et al., 1999). Both these processes increase the amount of AChE when and where it is needed. As AChE-R mRNA is significantly less stable than AChE-S mRNA (Chan et al., 1998; Luo et al., 1999), any stabilizing effect should significantly favor the R-variant. This should be of particular interest in the context of the routine use of anti-AChEs in the treatment of AD patients (Palmer, 2002). Indeed, AChE-R was recently shown to accumulate in the cerebrospinal fluid of AD patients treated with anti-AChEs (Darreh-Shori et al., 2002). The variable efficacy of such agents among individuals may therefore reflect differential capacities to induce transcriptional activation and/or stabilization of AChE mRNA.

Cholinergic neurotransmission and the biological role of acetylcholinesterase

AChE has long been recognized for its physiologically essential role in terminating cholinergic neurotransmission. It hydrolyzes the neurotransmitter, ACh, which is released from pre-synaptic sites and diffuses through the synaptic cleft to ACh receptors, which propagate the nerve impulse or initiate muscle contraction. Recent research, however, has indicated that AChE may serve additional functions. Early hints at non-classical roles for AChE came from its presence in non-

neuronal tissues such as the meninges (Razon et al., 1984), blood vessel endothelia (Pakaski and Kasa, 1992) and glia (Karpel et al., 1994). Human *ACHE* expression was also observed in primary carcinomas (Zakut et al., 1988), placental chorionic villi (Zakut et al., 1991) and embryonic bone (Grisaru et al., 1999a). Certain biological actions of AChE in these settings may require the protein's catalytic activity (see below). However, microinjection and transfection studies have demonstrated that recombinant AChE that had been engineered to be catalytically inactive, can still play a role in neurite growth (Grifman et al., 1998; Sternfeld et al., 1998a) and astrocyte development (Karpel et al., 1996).

Compensatory mechanisms as suppressors of stress symptoms

If, indeed, AChE over-production is generally associated with long-term stress responses, the question arises, How does the brain handle the resultant state that is induced by elevated ACh hydrolysis? The obvious answer is, by initiating compensatory mechanisms that would elevate the cholinergic state to retrieve functional balance. Similar compensatory mechanisms are generally assumed to enable the extended pre-symptomatic stages that accompany neurodegenerative disease, e.g. AD and PD (Zigmond, 1997). Neurodeterioration, in this view, would be due to inadequate compensation for consequences of both inherited defects and stress. Elevation of the cholinergic status would be possible only if sufficient numbers of viable cholinergic neurons were available. Indeed, animal models are known that develop cognitive impairments at relatively early ages due to inherited loss of cholinergic forebrain neurons (Sago et al., 1998; Zigmond, 1997).

ACHE *genetic and post-translational sources of variation*

In contrast to the wide spectrum of post-transcriptional variations of AChE, genomic polymorphisms in the human *ACHE* gene are rare, and those that have been discovered have no known effect on enzyme properties. Histidine substitution for asparagine at position 322 creates the YT_b blood group, indicating that this residue is part of an exposed epitope in erythrocytic AChE (AChE-E). The frequency of the polymorphism is higher in Middle East, as compared with European populations (Levene et al., 1987), but the H322N mutation has no effect on the catalytic properties of the enzyme, nor does a polymorphism that changes a P446 codon in the mRNA, but not the protein sequence of the enzyme (Bartels et al., 1993; Ehrlich et al., 1994). The negligible polymorphism in the AChE protein, in comparison with the abundant mutations in the homologous BuChE (see below), was taken to reflect the vital necessity of a functional AChE. However, there are polymorphisms in up-stream regulatory regions of *ACHE* (Grant et al., 2001; Shapira et al., 2000b). By affecting the level of gene expression, or the possibilities for alternative splicing of the mRNA, these polymorphisms can have important physiological consequences.

Environmental stress on AChE mitigated by butyrylcholinesterase

Since its discovery, AChE has been known as the enzyme that hydrolyzes the neurotransmitter, ACh. The biological role of the AChE-homologous enzyme, BuChE, has long been problematic. It has been postulated that BuChE is a back-up for AChE, and in the very special case of the AChE-knockout mouse, as mentioned, it may be BuChE that in fact performs such a function. Nevertheless, in the real world of Nature, there are no known cases of human AChE mutations that abolish its activity, which is a powerful message that AChE serves an irreplaceable function. It must be mentioned, however, that there are mutations of the AChE-S-anchoring protein, which

result in end-plate AChE deficiencies (Ohno et al., 2000). Another role proposed for BuChE is as a molecular decoy that absorbs anti-AChEs that may find their ways into the body and minimize this source of stress on the organ systems that depend on a functional AChE. AChE and BuChE have overlapping specificities for substrates and inhibitors, with BuChE being somewhat more promiscuous. In consequence, just about every anti-AChE is also an anti-BuChE. The environment contains many and varied anti-AChEs, ranging from the anatoxins, natural organophosphate poisons of glue-green algae (Carmichael, 1994) and the abundant glycoalkaloids of *Solanaceae* (potatoes, tomatoes, aubergines) to the toxins of snake venoms, e.g. fasciculin (Marchot et al., 1997). An anti-AChE entering the body will react with serum BuChE (and, for that matter, AChE-E on erythrocyte membranes) before ever coming into contact with AChE-S at NMJs or brain synapses. The individual is thus protected by the ability of BuChE to adsorb AChE inhibitors. Consistent with its being, in effect, a molecular decoy for AChE, are the prominence of BuChE in the serum and its capacity to react quickly with a wide spectrum of compounds. Furthermore, some polymorphisms of the *BCHE* gene render carriers increasingly susceptible to the ill effects of anti-AChE exposure. This polymorphism of *BCHE* has been extensively surveyed, originally by study of the variant-characteristic susceptibility to inhibitors of the serum activity (Kalow and Genest, 1957), more recently by molecular genotyping (La Du et al., 1990). *BCHE* mutations are very unevenly distributed around the world, with dramatically high or low frequencies found especially in historically isolated ethnic groups, possibly reflecting genetic founder effects. The different BuChE variants and their frequencies may also reflect an evolutionary adaptation to local environmental factors. Because BuChE mutants offer varying protection against anti-AChEs, carriers of these mutations may be more vulnerable than non-carriers to anti-AChEs and may show exaggerated responses when exposed. An extension of this idea, in conjunction with the similarity of chemical and other stressors, is increased vulnerability of BuChE mutation carriers to late-onset diseases. In the case of AD, some have found such an association (Lehmann et al., 1997; Lehmann et al., 2000), but others have not (Brindle et al., 1998).

Non-classical biological roles of acetylcholinesterase

Non-classical actions of AChE, i.e. those not associated with hydrolysis of ACh at the synapse or NMJ, have been reported by a number of laboratories, among them, those of M.E. Appleyard (Appleyard, 1992), John Bigbee (Bigbee et al., 1999), W. Stephen Brimijoin (Koenigsberger et al., 1997), Susan Greenfield (Greenfield, 1996), Paul Layer (Layer, 1996) and David Small (Small et al., 1996). It is still a challenge to integrate the disparate, but not contradictory, findings of these research groups into a coherent view of the biology of AChE. Recent discoveries may indicate a biochemical basis of at least some of these seemingly anomalous effects. There is a homology among AChE and other members of the α/β fold family of proteins, including the neurotactins, which are involved in cell-cell adhesion. The conserved domain of neurotactins may be exchanged for AChE and still retain cell-cell interaction (Darboux et al., 1996). Moreover, genetic inactivation of AChE does not prevent its neurite growth-promoting activity (Grifman et al., 1998; Sternfeld et al., 1998a). The mammalian non-catalytic AChE-homologous proteins, the neuroligins reside in excitatory synapses (Song et al., 1999), and are known to bind neuronal cell surface proteins, the neurexins (Nguyen and Sudhof, 1997); neurexins, neurotactins and neuroligins are transmembrane proteins with C-terminal cytoplasmic tails which could enable signal transduction through the binding of PDZ domain proteins, e.g. membrane-associated guanylate kinases (MAGUKs) (Ponting et al., 1997), and thus provide the molecular basis of the

complex consequences that are characteristic of these cell-cell interactions. Many in the research community are now open to the recognition of non-cholinergic roles of AChE (Botti et al., 1998), and more such functions will doubtless be identified in the near future.

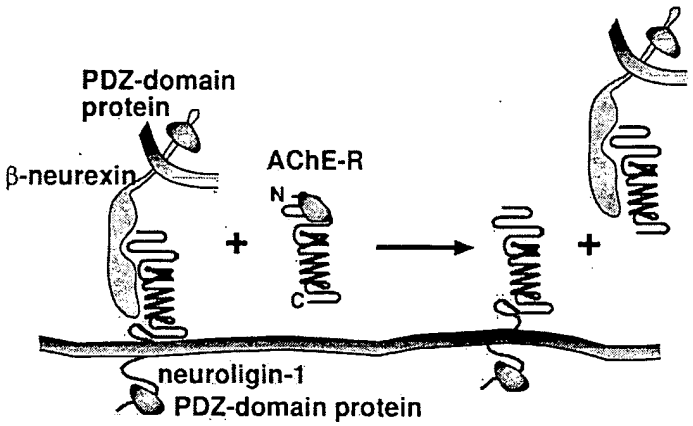


Fig. 2. Proposed molecular basis of non-catalytic properties of AChE. Cell-cell interactions are mediated by the interaction of membrane-bound neuroligin-1 with membrane-bound β-neurexin. If neuroligin-1 is displaced from β-neurexin by the homologous (Tsigelny et al., 2000), soluble, AChE-R, the cell-cell interaction is broken. This is likely to modify the properties and/or intracellular signaling activities of PDZ domain proteins that interact with both neuroligin-1 and β-neurexin, with predictable effects on excitatory synapse activities. When fully understood, this version may prove to have been too simplistic, as it cannot be readily reproduced in cultured neurons (Scheiffele et al., 2000), but the elements of it seem safely established.

Compensation for neuron loss by increased neurite outgrowth can delay the symptoms of neurodegeneration. Various studies support the notion of AChE's participation in such processes. Studies of the morphogenic roles of AChE have been facilitated by the construction of transgenic mouse lines that over-express a specific AChE variant, AChE-S (TgS) (Beeri et al., 1995) or AChE-R (TgR) (Sternfeld et al., 1998b), by the use of stably transfected cell lines (Bigbee et al., 1999; Grifman et al., 1998; Koenigsberger et al., 1997). and primary neurons that express and produce small quantities of a recombinant variant (Sternfeld et al., 1998a). In several of these model systems, human AChE-R emerged as having effects distinct from those of AChE-S. In cultured glioblastoma cells, over-expressed AChE-R confers a phenotype of small, round, rapidly dividing cells as opposed to the AChE-S phenotype of process growth (Karpel et al., 1996; Perry et al., in press). Antisense suppression of AChE mRNA in neuroblastoma cells was associated with complete loss of neuritogenesis, which was retrieved by re-transfection with AChE-S (Koenigsberger et al., 1997). Similar results were obtained in PC12 cells where either AChE-R or the non-catalytic homolog, neuroligin, retrieved neurite growth following antisense suppression of AChE-R (Grifman et al., 1998).

The changes under stress in the levels of AChE variants further imply an altered ratio between AChE-R and AChE-S in the stressed nervous system. This highlights one of the key challenges in this field, namely the search for the physiological functions of the different splice variants. While

previous theories of AChE's involvement in neurophysiological activities were largely limited to cholinergic neurotransmission, its non-catalytic activities likely span many more circuits and brain regions. It would be very interesting to know, for example, if the soluble AChE-R monomers secreted under stress serve to modulate glutamatergic neurotransmission or affect the stress-induced changes in long- term potentiation (Vereker et al., 2000), a cellular function related to memory, or the facilitation of long-term depression (Xu et al., 1997), its opposite.

Animal studies

Acetylcholinesterase-R is over-produced under the influence of several stressors

Facilitation of the capacity for ACh hydrolysis provides useful short-term suppression of the cholinergic hyperexcitation that is associated with stress responses. This can prevent epileptic seizures, a known consequence of anti-cholinesterase exposure (Blanchet et al., 1994; Shih and McDonough, 1997), and head injury (Shaw, 2002). However, in the long run, these forms of stress or trauma – acute psychological stress, exposure to anti-AChEs, head injury – all can lead to delayed neurodeterioration. Further studies will be required to determine whether the association of AChE-R with these physiological conditions reflects a causal relationship to neurodeterioration, or whether the expression of AChE-R is a protective mechanism that is not always sufficient to prevent the deterioration.

To begin to address these questions we examined stress-induced and stress-related neuropathological and behavioral parameters in mice with transgenic over-expression of various AChE isoforms. As described below, TgS mice exhibit impairments in learning and memory (Beeri et al., 1995), which may be partially accounted for by the biochemical alterations in their brains. TgS mice adapt to the high levels of AChE by increased synthesis of high affinity choline transporter (pre-synaptic choline uptake) and acetyl cholinetransferase (ACh synthesis). The rather counter-intuitive result is an unchanged level of ACh in awake mice; under halothane anesthesia the TgS ACh levels were lower than in parent strain mice, attesting to the transient nature of these compensatory effects (Erb et al., 2001). The modified AChE-R/AChE-S ratio may induce persistent changes in the CNS. Exposure of TgS mice to acute levels of the anti-AChE, diisopropylfluorophosphonate (DFP), failed to induce AChE-R over-production in their intestinal endothelium, an exposure response that occurred readily in the parent FVB/N strain (Shapira et al., 2000b). The high level of AChE in brains of TgS or the altered AChE-S/AChE-R ratio may render these mice particularly vulnerable to the long-term consequences of acute stress. The TgS mice make an intriguing model in which to study the roles of AChE and cholinergic signaling in mammalian stress responses, but because several components of the cholinergic system have been perturbed, a model with which it is necessary to perform a large number of control experiments.

Neuronal over-expression of AChE is associated with neuropathological and psychopathological stress responses

As mentioned, stress alters features of cholinergic neurotransmission. Superimposed on this are often the effects of aging and neurodegenerative diseases (Rehman and Masson, 2001). We first explored the specific contribution of variant AChE mRNA transcripts to endurance of neuronal properties with time. The brains of TgS mice were tested by high resolution *in situ* hybridization using cRNA probes selective for each of the two major brain variants of AChE, AChE-S and AChE-R. Excessive AChE-S mRNA was observed in TgS mice, as compared with non-

transgenic controls. This was consistent with the expected cumulative contributions of the transgenic human and the host mouse *ACHE*. Over-production of mouse neuronal AChE-R mRNA in these TgS mice was different in the various neuron subtypes, yet significantly higher than that in control mice. This suggested an inherited predisposition to constitutive AChE-R over-production in certain neurons within the brains of TgS mice. Moreover, the over-produced R transcripts travel more readily to neurites in TgS brain and even more dramatically following head injury or exposure to anti-AChEs (Meshorer et al., 2002). Because AChE-R over-production is associated with stress, a well as with hypersensitivity to both cholinergic agonists and antagonists (Meshorer et al., 2002), this further called for evaluating its neuroanatomical and behavioral manifestations.

AChE participation in stress-induced neuropathologies: neuroanatomical manifestations

Stress is associated with pathological changes in the brain (McEwen, 1999). To explore the progressive neuroanatomical changes associated with alterations in cholinergic balance, we employed our TgS mice. The dendritic arbors of adult transgenic mice became progressively smaller than those of adult controls. These findings, in conjunction with the impairments in both working and reference memory that are displayed by TgS mice (Beeri et al., 1997), demonstrate in these mice 3 hallmarks associated with senile dementia, namely, progressive cognitive failures, enhanced high affinity choline uptake and late-onset cessation of dendrite branching. It should be emphasized that TgS mice do not display amyloid plaques, and thus lack the defining hallmark of AD. These mice, therefore, display a more specifically stress-related pattern of neurodeterioration.

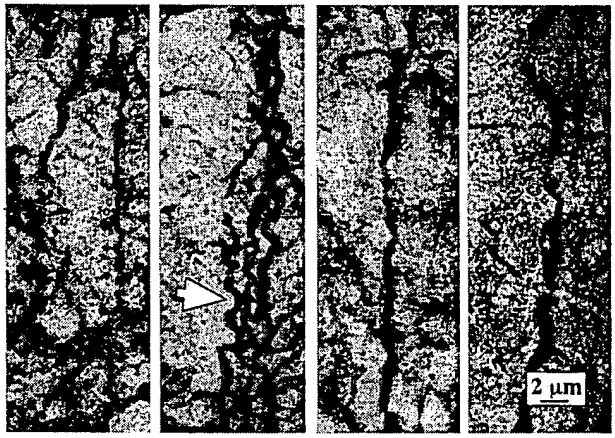


Fig. 3. Neuropathologies induced by transgenic AChE-S. Sections of cortex were taken from normal, TgS, transgenic human AChE-Sin (an inactive engineered variant of AChE-S in which a 7-residue sequence interrupts the active site), and AChE-R. The pathological "corkscrew" structures (red arrow) appear in the brains of the TgSs, much more than in the brains of control, AChE-Sin or TgRs. (Sternfeld et al., 2000)

To assess the specific neuroanatomical effects of AChE-R, we studied TgR mice. These mice were found to display lower levels than controls of stress-associated hallmarks of pathology (accumulation of heat shock protein HSP70, presence of reactive glia and curled neurites) (Corso et al., 1997; Hamos et al., 1991; Kubo et al., 1998; Li et al., 1998), whereas TgSs exhibit accelerated stress-related pathologies (Sternfeld et al., 2000). TgS mice, which over-produce AChE, apparently to their limit, cannot respond by further AChE production and are therefore exceptionally sensitive to closed head injury (Shohami et al., 2000), switch in circadian light/dark cycle (Cohen et al., 2002) and exposure to AChE inhibitors (Shapira et al., 2000b). This suggests a physiological relevance of AChE's alternative splicing in response to stressors. To this end, TgS mice also over-produce the host AChE-R, which is likely to be a systemic response to the excess AChE-S at cholinergic synapses. That AChE-R over-production is, itself, a liability to the organism is evident from the retrieval of rapid recovery from head injury following the destruction of AChE-R by antisense mechanisms (Shohami et al., 2000). In defining the stress-induced changes in AChE, is the challenge to locate the effect in the chain of stress-initiated events. Table 1 lists some of the studied phenotypes and their outcomes.

Table 1. Stress-inducing physiological phenotypes associated with cholinergic impairments

Insult	outcome	reference
Forced swim (psychological)	hippocampal hyperexcitation	Friedman et al., 1998
Light/dark switch (physiological)	enhanced locomotion	Cohen et al., 2002
Anti-cholinesterase exposure	impaired working memory	Kaufer et al., 1999
Head injury	impaired motor coordination	Shohami et al., 2000

Behavioral manifestations of stress

Behavioral differences between TgS and control mice were first sought by telemetric recording of locomotion patterns, both under basal conditions, as well as following the stress of a switch in the circadian light/dark cycle. Under normal conditions, naïve TgS mice displayed close to normal locomotion behavior, similar to that of naïve mice from the non-transgenic strain. However, their locomotor response to stress, either in their cages or in an elevated plus maze, was distinct from that of the parent strain. We also assessed the effects of AChE over-expression in these mice on memory functioning, which is known to be markedly affected by exposure to various physical and psychological stressors. This issue is summarized in Table 2 and detailed below.

Table 2. Behavioral stress correlates in TgS mice

behavioral test	stress-relevant phenotype	reference
Morris water maze	impaired acquisition of spatial information	Beeri et al., 1995; Beeri et al., 1997
Social exploration	deficient working memory (extended sniffing time)	Cohen et al., 2002
Elevated plus maze	reduced anxiety (prolonged open arm time)	Erb et al., 2001
Locomotion	aimless hyperactivity after light/dark switch	Cohen et al., 2002

Response to circadian shift:
Cholinergic neurotransmission circuits are known to be subject to circadian changes (Carlson, 1994) and control the sensorimotor cortical regions regulating such activity (Fibiger, 1991). Conversely, a stressful shift in circadian cycle is known to produce considerable impairment of the locomotor behavior of both animals and humans (Lightman et al., 2000; Weibel et al., 1999). To assess the general responsiveness of normal and TgS mice to this stressor, we recorded their locomotor activity, using telemetric transmitters, implanted in the peritoneal cavity. Movement was monitored by a sensor to estimate distance the animal moved over several days.

Under routine conditions, both genotypes displayed similar home cage activity. Their circadian rhythms included, as expected, significantly more frequent and pronounced locomotor activity during the early part of the dark phase of the circadian cycle. Seventy-two hours following reversal of the light/dark phases, both genotypes lost most of the circadian rhythm in their locomotor activity. This response is common to most rodents, as reported by others (Hillegaart and Ahlenius, 1994). However, normal and TgS mice subjected to a circadian light/dark shift presented distinctive behavioral patterns. The parent strain, similarly to other mammals, presented general reduction in post-shift locomotor activity. In sharp contrast, TgS mice showed a general increase in post-shift activity, both during the dark and the light phases. In particular, activity in the dark phase of the reversed cycle was significantly greater in TgS compared with control mice. These findings indicate that adjustment to the circadian insult was markedly impaired in TgS mice, suggesting that these mice display a genetic predisposition to abnormal responses to changes in the circadian rhythm, and perhaps other stresses, as well.

Findings of others (Winslow and Camacho, 1995) and the intensified response of TgS mice to the stress of a circadian switch suggested that the excess of AChE is the cause. The variable nature of the excessive locomotor activity in individual TgS mice indicated an acquired basis for its extent and duration. A potential origin of such heterogeneity could be the increased amount of neuronal ChAT and mAChE-R mRNAs in the sensorimotor cortex and hippocampal neurons. As mentioned above, both psychological and physical stressors induce neuronal AChE-R over-production. Transcriptional activation and shifted alternative splicing thus prevent excess accumulation of AChE-S, the C-terminus of which includes a region with reported neurotoxic properties (Greenfield and Vaux, 2002). However, the exaggerated stress responses, such as the intense locomotor response to the mild stress of a circadian switch can be expected to exacerbate the hypocholinergic state of these already compromised animals, as is also indicated from their short-term response to tacrine (Cohen et al., 2002; Gheusi et al., 1994).

Transient antisense suppression of transgenics' locomotor activity further substantiates the involvement of AChE-R in the circadian light/dark shift-associated hyperactive response. To assess this assumption, TgS mice were injected intraperitoneally with 50 μg/Kg of a 3' terminally 2'-O-methyl protected antisense oligonucleotide, EN101, that suppresses *de novo* production of mouse AChE-R (Cohen et al., 2002; Shohami et al., 2000). The difference between pre- and post-treatment locomotor activity was calculated for each animal, following EN101 or saline injection. TgS mice presented only a transient decrease in locomotor activity following EN101 treatment. This called for using a different behavioral paradigm, one that would avoid transmitter implantation and allow direct EN101 injection into the brain.

AChE over-expression and memory functioning.
The finding that AD is associated with premature death of cholinergic neurons, initially within the basal forebrain, and the limited efficacy of anti-AChE drugs in ameliorating these deficits, suggested that excess AChE produces cognitive impairments. However, stress is also associated with cognitive disturbances (Lupien and Lepage, 2001). Therefore, stress-induced increases in AChE expression and catalytic activity, may explain the stress component of cognitive impairment in the demented brain. The cognitive failure of TgS mice supports this assumption, as detailed below.

Progressive spatial memory decline as monitored in the Morris water maze:
To examine their cognitive functioning, TgS mice and wild-type controls were tested in the Morris water maze. This test assesses spatial memory, which is known to deteriorate with age; however, the age-dependent deterioration in spatial memory was rapidly accelerated in TgS mice (Beeri et al., 1995), reaching a level of total failure in young adults (Beeri et al., 1997).

The reduced dendritic fields in the brains of these mice (Beeri et al., 1997) might represent the structural correlate of their spatial memory impairment. This supports previous studies that have frequently revealed neuronal damage or atrophy, mirroring cognitive dysfunction in demented animals and humans. While it is not yet clear whether the limited dendritic arbors in TgS mice reflect enhanced pruning or a suppressed growth of neurites, the thinned neural network that this entails mimics a parallel phenomenon in the brain of AD patients (Ruppin and Reggia, 1995). The impairments in the Morris water maze performance in TgS mice thus suggest that prolonged AChE over-expression is associated with a progressive decline in learning and memory.

AChE and the disruption of learning and memory
The delayed consequences of stress and neurodegeneration are both known to involve impairment of other aspects of learning and memory, in particular those that related to individual relationships. To test whether this aspect of behavior, as well, is associated with AChE regulation, we assessed the memory functioning of TgS mice in the social recognition test, which is based on olfactory perception (Dantzer et al., 1990).

EN101 improvement of short-term memory increases in efficacy and duration in animals with severe pre-treatment impairments
The effects of stress depend both on genetic and environmental elements, and even the inbred TgS mice present heterogeneous behavioral characteristics. To examine the effect of EN101 on social recognition, and its correlation with pre-treatment symptom severity, TgS mice were divided into three groups, and those exhibiting short or long exploration of a familiar juvenile were selected based on a baseline social recognition test. Social recognition was tested again 1, 3 and 6 days following two intracerebroventricular injections of EN101, separated by 24 hr. As expected, there was a significant overall difference between the short and the long groups in exploration time. However, post-hoc tests revealed that these groups differed significantly only during the pre-treatment day, and not after the EN101 treatment. Furthermore, within the long, but not the short explorers group, social exploration of the 'same' juvenile was significantly reduced 1 day after the EN101 injection, with progressive increases in social exploration time during the 5 subsequent days. Because of the pre-treatment differences, the severely impaired animals sustained a certain level of improvement even at the sixth post-treatment day (i.e., even

on this day there was no resumption of the pre-treatment difference between the short and long explorers). This experiment thus demonstrated both the efficacy and the reversibility of the antisense treatment, and emphasized its exceedingly long duration, especially in animals with severe pre-treatment impairments and in comparison to the short-term efficacy of tacrine (Cohen et al., 2002).

Antisense AChE-R mRNA suppression selectively reduces brain AChE-R protein
To further prove the putative role of AChE-R in mediating the impaired social recognition, we conducted a second experiment, in which mice completed a social recognition test before and after two injections with either EN101 or a sequence specificity control. 24 hr following the second test, their brains were removed and AChE-R expression was assessed.

As found in the first experiment, Tg mice with long pre-treatment explorative behavior displayed a significant improvement in social exploration of the 'same' juvenile 24 h following the second treatment with EN101, but not with the irrelevant AS-ON targeted to BuChE mRNA. Control mice with either long or short pre-treatment social exploration showed no response to either EN101 or ASB, perhaps reflecting a limitation in the resolution power of these behavioral tests.

Immunodetected AChE-R protein levels were significantly lower in EN101 treated mice as compared with ASB treated mice, regardless of their genotype or pre-treatment behavior pattern. In contrast, densitometric analysis of immunodetected total AChE protein (detected by an antibody targeted to the N-terminus, common to both isoforms) revealed essentially unchanged signals. Together, these findings attest to the selectivity of the antisense treatment for treating AChE-R over-expressing animals and its sequence-specificity in reversing the AChE-R induced impairment of behavior. The outcome of this second experiment has also provided a tentative explanation of the long duration of the antisense effect, in that disrupted function appears to be associated with higher levels of AChE-R. So long as AChE-R remains below a threshold level, function remains normal, even if the antisense agent is no longer present. AChE-R expression, thus, presents a relatively wide safety margins, above which it causes deleterious effects.

Human Studies
Gulf War syndrome and penetrance of the blood-brain barrier
Cholinergic pathways are involved in controlling numerous peripheral functions, e.g. neuromuscular activity, salivation, intestinal functions and lacrymation. Over the years, this led to the development of drugs aimed at controlling these functions. To avoid unwanted effects over higher brain functions, these drugs were designed to remain in the circulation, where they are held thanks to the integrity and efficiency of the blood-brain barrier (BBB) (Rubin and Staddon, 1999). A notable example is pyridostigmine bromide (PB), used for the past 40 years for treating patients with myasthenia gravis, an autoimmune syndrome that causes debilitating muscle fatigue (Berrouschot et al., 1997). During the 1991 Gulf War, injection of PB was a prophylaxic treatment of soldiers, in anticipation of chemical warfare, to transiently block AChE and protect it from permanent inhibition by nerve agents. This treatment was based on clinical studies that demonstrated its effectiveness and showed side effects that were strictly limited to peripheral symptoms (Beck et al., 2001). However, impairments in higher functions of treated soldiers and subsequent symptoms that appeared to originate in the CNS led to questions regarding the ability of PB to remain peripheral under the stresses that were associated with the war (Golomb, 1999).

This, in turn, provoked further studies that explored behavioral changes under PB administration. It was thus found that PB modified the acoustic startle response (Servatius et al., 1998), locomotor activity in an open field (Kant et al., 2001), hand and eye coordination (Wolthuis et al., 1995), the capacity to press a lever in a delayed reinforced manner (van Haaren et al., 1999) and visual discrimination properties (Liu, 1992). A relatively simple explanation of these behavioral effects would be that the stress associated with PB injection modifies the properties of the BBB and enabled PB penetrance of the brain, in spite of the cationic group that had been introduced into this carbamate to prevent penetrance through the BBB. This was, indeed, the working hypothesis of a study (Friedman et al., 1996) that reported efficient inhibition of brain AChE by intraperitoneally injected PB into forced swim-stressed, but not naïve, mice. However, the complete picture is far from simple, as other studies (with other stressors and other rodents) reported no inhibition of brain AChE under systemic administration of PB (Cook et al., 1988; Stitcher et al., 1978). The later-reported transcriptional feedback response, including AChE over-production under exposure to anti-cholinesterases (Kaufer et al., 1998) offers a tentative explanation of this inconsistency. Yet more recently, the lethality of PB in animals was reported to be increased (Chaney et al., 1999; Chaney et al., 2000) and its capacity to inhibit brain AChE facilitated, when co-injected with permethrin or N,N-diethyl-*m*-tolumide (Abou-Donia et al., 2001; Abou-Donia et al., 1996). While it is difficult to compare these studies to one another, because they used different stresses or animals, it seems possible to conclude that under some circumstances stress can cause permeabiliztion of the BBB.

Corticotropin releasing hormone (CRH), a 41-residue peptide originating in the hypothalamus, apparently has a central role in response to stress. It has long been known to cause release of adrenocorticotropin from the pituitary into the bloodstream, where it travels to the adrenal cortex to promote synthesis and secretion of cortisol, which is the source of many other stress responses, among them activation of *ACHE* gene expression. More recently, CRH, acting with mast cells, has been shown to increase permeability of the BBB (Esposito et al., 2002; Esposito et al., 2001). In a dramatic demonstration of the effect of stress, Relyea and Mills found that the anti-AChE pesticide, carbaryl, was 2 to 4 times more lethal to amphibians when they were exposed to the stress of predatory cues (Relyea and Mills, 2001). This may be an additional demonstration of the effect of stress on the BBB.

A pathological case of AChE in Alzheimer's disease fibrils and plaques

We presume that the interactions of AChE with other cell elements is a normal physiological function, but AChE over-expression under stress may have long-term consequences that can potentially affect the initiation and rate of progress of neurodegenerative disease. Indeed, AChE has been found in the plaques and tangles to which are attributed the neurotoxicity of AD *in vivo* (Luth et al., 2001; Mesulam et al., 1992; Sberna et al., 1997). AChE binding to β-amyloid has been observed both *in vitro* (Inestrosa et al., 1996) and *in vivo* (Inestrosa et al., 2000), and was reported to enhance the development of amyloid plaques. Recently, we tested the assumption that AChE over-expression facilitates the progress of AD by mating TgS mice with transgenics that express the amyloid protein with the Swedish double mutation, which increases the risk for early-onset AD. As predicted, the inherited AChE over-expression induced the appearance of amyloid plaques considerably earlier than in the parent strain, attesting to the potential effect of stress on the age of onset of neurodegenerative processes (Rees et al., in press).

Cholinesterase genetics and stress responses

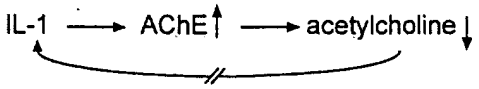
An up-regulation of AChE activity occurs in response to exposure to anti-AChEs (Fullerton and Ursano, 1990; Somani et al., 2000). However, in some humans, there is a higher than usual basal level of *ACHE* expression. As in most circumstances, the individual shows no ill effects, there is an adaptation to this state. This may involve an increased level of ACh receptors, similarly to TgS mice (Perry et al., 2000), or increased high affinity choline transporter, also shown in TgS mice (Erb et al., 2001). It was hypothesized that similarly to TgS mice, individuals with constitutive AChE over-expression would be unable to respond appropriately to exposure to anti-AChEs and that their *ACHE* gene would contain some clues as to the cause. Therefore the genomic DNA from 340 subjects, including several who suffered cholinergic symptoms under anti-AChE exposure, was analyzed, with special attention to a region of the promoter sequence that was rich in transcription factor binding elements. Two adjacent mutations in a distal upstream enhancer domain of the human *ACHE* gene were discovered in heterozygous carriers (Shapira et al., 2000a): a 4-bp deletion and a single nucleotide substitution. The deletion, identified in a woman who presented acute hypersensitivity to pyridostigmine, was found in transfected cells to constitutively increase AChE expression by abolishing 1 of 2 adjacent HNF3 binding sites. Because the deletion confers a gain of function of *ACHE*, the trait is dominant; the substitution impairs a glucocorticoid receptor binding site. Further studies will be required to find whether this trait is also associated with increased risk for over-sensitivity to stress.

Peripheral/central interactions: transfer of stress signals through cholinergic pathways

Recently, a "cholinergic anti-inflammatory pathway" has been identified in which cholinergic signaling through the efferent vagus nerve modulates the mammalian inflammatory response (Bernik et al., 2002; Borovikova et al., 2000; Tracey et al., 2001). ACh, the principal vagal neurotransmitter, significantly attenuated the release of the proinflammatory, tissue necrosis factor-α (TNFα), interleukin (IL)-1β, IL-6 and IL-18, but not the anti-inflammatory cytokine IL-10, in lipopolysaccharide-stimulated human macrophage cultures and in live rats. A parallel process in the brain, or indeed in other leukocytes, has not yet been explored. An interesting point of this observation is that ACh in the blood is given a physiological role, and as a corollary, the AChE in blood, notably that on the surface of erythrocytes, is brought into question. However, erythrocytic AChE changes only very slowly, which seems to disqualify it from a role in inflammatory responses; AChE-R, being soluble and having a short half-life is a more likely regulator of the responses to inflammatory challenges, as well as a controller of the anxiety associated with the inflammatory responses (Danzer, 1999; Reichenberg et al., 2001).

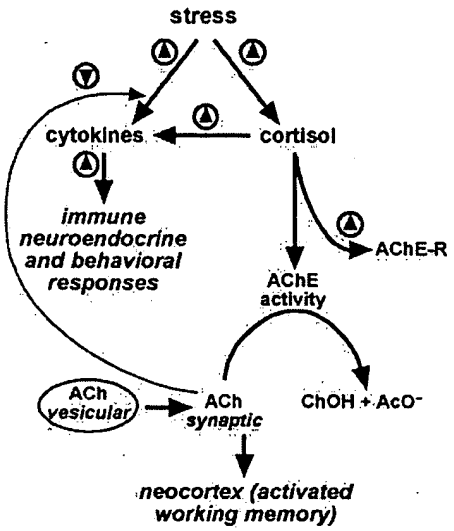
In light of reports that stress, both in humans and in animals, involves increased production of pro-inflammatory cytokines, e.g. IL-1 (Maes et al., 1998; Nguyen et al., 1998; Spivak et al., 1997), and that IL-1 causes AChE over-production in PC12 cells (Li et al., 2000), we postulate the following relationships (Scheme 1): IL-1 induction of AChE over-expression suppresses ACh, ablating the interference by ACh in IL-1 production, a cycle that may explain prolongation of stress responses (Scheme 1).

Scheme 1



A natural extension of this peripheral concept would be to predict that AChE-R levels should be pivotal for regulation of ACh in both the peripheral and central nervous systems. Thus, stress-induced elevation in cortisol levels results in elevated neuronal AChE production (Meshorer et al., 2002). This would reduce ACh and elevate cytoine production (Scheme 2). According to this model, in addition to the direct suppressive effects of cortisol on cytokine production (Marx, 1995), it also produces activation of cytokine production, by an indirect cholinergic-mediated mechanism. This indirect route may explain some of the permissive and pro-inflammatory actions of glucocorticoids (Brooke and Sapolsky, 2002; Munck and Naray-Fejes-Toth, 1994; Wilckens and De Rijk, 1997).

Scheme 2



Shown are the cellular and biochemical events to which we attribute predicted stress-associated changes. Stress induces the release of cytokines and cortisol. Cytokines elevation is associated with immune, neuroendocrine and behavioral responses (Tokuyama et al., 2000). Cortisol induces AChE-R production, which should elevate (▲) AChE plasma activity. Vesicular ACh is released into the synaptic cleft, where it affects neuronal electrophysiology and may improve working memory (Furey et al., 2000). In the periphery, ACh acts to suppress cytokines production in macrophages (upward curved arrow).

AChE-R accumulation is transient because this enzyme and its mRNA are relatively unstable. Therefore, the cognitive effects of inflammation would be expected to be limited to the duration of cortisol's presence in the circulation and perhaps several hours after, but not much longer. This theory is compatible with previous findings in humans (Yirmiya et al., 2000) and with our on-going studies (O. Cohen, unpublished data).

The future of acetylcholinesterase in stress studies

Anti-AChE therapies

Unwanted long-term sequelae of exposure to anti-AChEs may possibly relate to the non-catalytic functions of AChE. However, the currently approved drugs for the treatment of AD patients are

all designed to suppress the catalytic activity of AChE (Giacobini et al., 2002). While this is aimed at redressing the imbalance in the cholinergic system caused by a selective loss of cholinergic neurons, these drugs do not reverse the underlying course of the disease. Moreover, recent work of others (Nitsch et al., 1998) and our own (Darreh-Shori et al., 2002) suggests that at least some of the effects of these drugs are associated with the feedback response leading to AChE-R accumulation. This emphasizes the requirement to reach a desired balance between AChE-S and AChE-R, rather than merely block AChE catalysis, and calls for novel approaches to gene regulation. The conflicting demands of therapy for treatment of exposure to anti-AChEs also include the short-term necessity of re-establishing AChE's role in cholinergic neurotransmission and the long-term need to prevent an exaggerated rebound increase in AChE-R levels.

As the role of AChE-R in stress responses is brought to light, it is becoming apparent that a key to regulating these responses is manipulation of the levels of this protein. It will be a challenge to devise therapeutic strategies that will regulate it, while leaving normal cholinergic neurotransmission unaffected. One such strategy uses an antisense reagent to specifically destroy the mRNA that encodes that variant. Although the antisense approach has been around for several decades, the mechanism of action of antisense oligonucleotides is incompletely understood. Nevertheless, whatever the molecular mechanisms there have been experimental and even clinical successes in using this approach (Orr, 2001). In our hands such an agent, EN101, has successfully aided recovery from closed head injury in mice (Shohami et al., 2000), retrieved functional working memory in TgS mice (Cohen et al., 2002) and reversed the symptoms of experimental autoimmune myasthenia gravis in rats (Brenner et al., in press). EN101 is now being tested in the clinic for treatment of human myasthenia gravis.

The cholinergic component of stress may confer bidirectional effects on cognitive functions
Behind the conventional view of stress responses reflecting a complex syndrome or disease, lies the fact that acute stress represents an extreme example of a natural process, enabling the adjustment to changing environment through neuronal plasticity. This is consistent with the fact that acute glucocorticoid administration, while impairing retrieval of long-term declarative memory (Kirschbaum et al., 1996), improves the working memory in humans (de Quervain et al., 2000). It is also compatible with the reports that cholinergic enhancement facilitates the increased selectivity of perceptual processing during working memory (Furey et al., 2000), and that higher cortisol values facilitate spatial memory in toddlers (Stansbury et al., 2000).

Concomitant with the bi-directional effects of stress on memory functions are remodeling changes in the dendritic arbors of neurons in various brain regions. These depend both on the type of stress and the brain region where they occur; for example, chronic immobilization stress induces dendritic atrophy and debranching in CA3 pyramidal neurons of the hippocampus, while pyramidal and stellate neurons in the amygdala exhibit enhanced dendritic arborization in response to the same stress (Vyas et al., 2002). Chronic, unpredictable stress, however, had little effect on CA3 neurons, but induced atrophy in bipolar neurons of the basolateral complex in the amygdala (Vyas et al., 2002). The dendritic atrophy observed in pyramidal cortical neurons of TgS mice (Beeri et al., 1997) demonstrates yet another version of this response, highlighting the fact that we know very little about the cellular and molecular differences among the responses to different kinds of stress.

This chapter would be incomplete without mention of the complex signaling cascades which are pivotal for the described stress responses. These likely involve changes in Ca^{++}-dependent protein kinase (PKC) activity and intracellular translocation (Young et al., 2002), especially PCKβII, which has been found essential for the contextual fear response, a characteristic consequence of acute stress (Weeber et al., 2000). A tentative link between this signaling cascade and the cholinergic feedback response to stress was indicated in a recent two-hybrid search for cellular binding partners of AChE-R, which revealed association of AChE-R with the PKCβII scaffold protein, RACK1 (Birikh et al., in press). The decreases in RACK1 in the brain of AD patients (Battaini et al., 1999) further support the yet elusive link between the stress load with which one is confronted and the onset of neurodegeneration.

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Bibliography

Abou-Donia, M. B., Goldstein, L. B., Jones, K. H., Abdel-Rahman, A. A., Damodaran, T. V., Dechkovskaia, A. M., Bullman, S. L., Amir, B. E., and Khan, W. A. (2001). Locomotor and sensorimotor performance deficit in rats following exposure to pyridostigmine bromide, DEET, and permethrin, alone and in combination. *Toxicol. Sci.*, 60: 305-314.

Abou-Donia, M. B., Wilmarth, K. R., Jensen, K. F., Oehme, F. W., and Kurt, T. L. (1996). Neurotoxicity resulting from coexposure to pyridostigmine bromide, deet, and permethrin: implications of Gulf War chemical exposures. *J. Toxicol. Environ. Health*, 48: 35-56.

Andres, C., Beeri, R., Friedman, A., Lev-Lehman, E., Henis, S., Timberg, R., Shani, M., and Soreq, H. (1997). Acetylcholinesterase-transgenic mice display embryonic modulations in spinal cord choline acetyltransferase and neurexin Iß gene expression followed by late-onset neuromotor deterioration. *Proc. Natl. Acad. Sci. U. S. A.*, 94: 8173-8178.

Appleyard, M. E. (1992). Secreted acetylcholinesterase: non-classical aspects of a classical enzyme. Trends Neurosci 15: 485-490.

Ashani, Y., Shapira, S., Levy, D., Wolfe, A. D., Doctor, B. P., and Raveh, L. (1991). Butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. *Biochem. Pharmacol.*, 41: 37-41.

Atanasova, E., Chiappa, S., Wieben, E., and Brimijoin, S. (1999). Novel messenger RNA and alternative promoter for murine acetylcholinesterase. *J. Biol. Chem.*, 274: 21078-21084.

Bartels, C. F., Zelinski, T., and Lockridge, O. (1993). Mutation at codon 322 in the human acetylcholinesterase (ACHE) gene accounts for YT blood group polymorphism. *Am. J. Hum. Genet.*, 52; 928-936.

Battaglia, M. (2002). Beyond the usual suspects: a cholinergic route for panic attacks. *Mol. Psychiatry*, 7: 239-246.

Battaini, F., Pascale, A., Lucchi, L., Pasinetti, G. M., and Govoni, S. (1999). Protein kinase C anchoring deficit in postmortem brains of Alzheimer's disease patients. *Exp. Neurol.*, 159: 559-564.

Beck, K. D., Zhu, G., Beldowicz, D., Brennan, F. X., Ottenweller, J. E., Moldow, R. L., and Servatius, R. J. (2001). Central nervous system effects from a peripherally acting

cholinesterase inhibiting agent: interaction with stress or genetics. *Ann. N. Y. Acad. Sci.*, 933: 310-314.

Beeri, R., Andres, C., Lev-Lehman, E., Timberg, R., Huberman, T., Shani, M., and Soreq, H. (1995). Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. *Curr. Biol.*, 5: 1063-1071.

Beeri, R., Le Novere, N., Mervis, R., Huberman, T., Grauer, E., Changeux, J. P., and Soreq, H. (1997). Enhanced hemicholinium binding and attenuated dendrite branching in cognitively impaired acetylcholinesterase-transgenic mice. *J. Neurochem.*, 69: 2441-2451.

Bergman, H., and Deuschl, G. (2002). Pathophysiology of Parkinson's disease: from clinical neurology to basic neuroscience and back. *Mov. Disord.*, 17 Suppl. 3: S28-40.

Bernik, T. R., Friedman, S. G., Ochani, M., DiRaimo, R., Ulloa, L., Yang, H., Sudan, S., Czura, C. J., Ivanova, S. M., and Tracey, K. J. (2002). Pharmacological stimulation of the cholinergic antiinflammatory pathway. *J. Exp. Med.*, 195: 781-788.

Berrouschot, J., Baumann, I., Kalischewski, P., Sterker, M., and Schneider, D. (1997). Therapy of myasthenic crisis. *Crit. Care Med.*, 25: 1228-1235.

Bigbee, J. W., Sharma, K. V., Gupta, J. J., and Dupree, J. L. (1999). morphogenic role for acetylcholinesterase in axonal outgrowth during neural development. *Environ. Health Perspect.*, 107, Suppl. 1: 81-87.

Birikh, K., Sklan, E., Shoham, S., and Soreq, H. (in press). Intensified stress-induced behavioral inhibition under "readthrough" acetylcholinesterase with the scaffold protein RACK1 and protein kinase. *Proc.* Natl. Acad. Sci. U. S. A..

Blanchet, G., Carpentier, P., Lallement, G., and Sentenac-Roumanou, H. (1994). [Prevention and treatment of status epilepticus induced by soman.] *Ann. Pharm. Fr.*, 52: 11-24.

Borovikova, L. V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G. I., Watkins, L. R., Wang, H., Abumrad, N., Eaton, J. W., and Tracey, K. J. (2000). Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature*, 405: 458-462.

Botti, S. A., Felder, C. E., Sussman, J. L., and Silman, I. (1998). Electrotactins: a class of adhesion proteins with conserved electrostatic and structural motifs. *Protein Eng.*, 11: 415-420.

Brenner, T., Hamra-Amitay, Y., Evron, T., Boneva, N., Seidman, S., and Soreq, H. (in press). The role of readthrough acetylcholinesterase in the pathophysiology of myasthenia gravis. *FASEB J.*

Brindle, N., Song, Y., Rogaeva, E., Premkumar, S., Levesque, G., Yu, G., Ikeda, M., Nishimura, M., Paterson, A., Sorbi, S., et al. (1998). Analysis of the butyrylcholinesterase gene and nearby chromosome 3 markers in Alzheimer disease. *Hum. Mol. Genet.*, 7: 933-935.

Brooke, S. M., and Sapolsky, R. M. (2002). Glucocorticoid exacerbation of gp120 neurotoxicity: role of microglia. *Exp. Neurol.*, 177: 151-158.

Burchfiel, J. L., and Duffy, F. H. (1982). Organophosphate neurotoxicity: chronic effects of sarin on the electroencephalogram of monkey and man. *Neurobehav. Toxicol. Teratol.*, 4: 767-778.

Camp, S., and Taylor, P. (1998). Intronic elements appear essential for the differentiation-specific expression of acetylcholinesterase in C2C12 myotubes. In: Proteins, B. P. Doctor, P. Taylor, D. M. Quinn, R. L. Rotundo, and M. K. Gentry (Eds.) *Structure and Function of Cholinesterases and Related*, Plenum Press, New York, pp. 51-55.

Carlson, N. R. (1994). Physiology of Behavior, Allyn and Bacon, Needham Heights.

Carmichael, W. (1994). The toxins of cyanobacteria. *Sci. Am.*, 270: 78-86.

Chan, R. Y., Adatia, F. A., Krupa, A. M., and Jasmin, B. J. (1998). Increased expression of acetylcholinesterase T and R transcripts during hematopoietic differentiation is accompanied by parallel elevations in the levels of their respective molecular forms. *J. Biol. Chem.*, 273: 9727-9733.

Chaney, L. A., Rockhold, R. W., Wineman, R. W., and Hume, A. S. (1999). Anticonvulsant-resistant seizures following pyridostigmine bromide (PB) and N,N-diethyl-m-toluamide (DEET). *Toxicol. Sci.*, 49: 306-311.

Chaney, L. A., Wineman, R. W., Rockhold, R. W., and Hume, A. S. (2000). Acute effects of an insect repellent, N,N-diethyl-m-toluamide, on cholinesterase inhibition induced by pyridostigmine bromide in rats. *Toxicol. Appl. Pharmacol.*, 165: 107-114.

Cohen, O., Erb, C., Ginzberg, D., Pollak, Y., Seidman, S., Shoham, S., Yirmiya, R., and Soreq, H. (2002). Neuronal overexpression of 'readthrough' acetylcholinesterase is associated with antisense-suppressible behavioral impairments. *Mol. Psychiatry*, 7: 874-885.

Cook, W. O., Beasley, V. R., Dahlem, A. M., Dellinger, J. A., Harlin, K. S., and Carmichael, W. W. (1988). Comparison of effects of anatoxin-a(s) and paraoxon, physostigmine and pyridostigmine on mouse brain cholinesterase activity. *Toxicon*, 26: 750-753.

Corso, T. D., Sesma, M. A., Tenkova, T. I., Der, T. C., Wozniak, D. F., Farber, N. B., and Olney, J. W. (1997). Multifocal brain damage induced by phencyclidine is augmented by pilocarpine. *Brain Res.*, 752: 1-14.

Dantzer, R., Tazi, A., and Bluthe, R. M. (1990). Cerebral lateralization of olfactory-mediated affective processes in rats. *Behav. Brain Res.*, 40: 53-60.

Danzer, R., Whollman, EE, and Yirmiya, R. (1999). Cytokines, Stress and Depression, Kluwer Academic/Plenum, New York.

Daoud, R., Da Penha Berzaghi, M., Siedler, F., Hubener, M., and Stamm, S. (1999). Activity-dependent regulation of alternative splicing patterns in the rat brain. *Eur. J. Neurosci.*, 11: 788-802.

Darboux, I., Barthalay, Y., Piovant, M., and Hipeau Jacquotte, R. (1996). The structure-function relationships in Drosophila neurotactin show that cholinesterasic domains may have adhesive properties. *EMBO J.*, 15: 4835-4843.

Darreh-Shori, T., Almkvist, O., Guan, Z. Z., Garlind, A., Strandberg, B., Svensson, A. L., Soreq, H., Hellstrom-Lindahl, E., and Nordberg, A. (2002). Sustained cholinesterase inhibition in AD patients receiving rivastigmine for 12 months. *Neurology*, 59: 563-572.

Dazzi, L., Motzo, C., Imperato, A., Serra, M., Gessa, G. L., and Biggio, G. (1995). Modulation of basal and stress-induced release of acetylcholine and dopamine in rat brain by abecarnil and imidazenil, two anxioselective gamma-aminobutyric acidA receptor modulators. *J. Pharmacol. Exp. Ther.*, 273: 241-247.

de Quervain, D. J., Roozendaal, B., Nitsch, R. M., McGaugh, J. L., and Hock, C. (2000). Acute cortisone administration impairs retrieval of long-term declarative memory in humans. *Nat. Neurosci.*, 3: 313-314.

Doctor, B. P., Blick, D. W., Caranto, G., Castro, C. A., Gentry, M. K., Larrison, R., Maxwell, D. M., Murphy, M. R., Schutz, M., Waibel, K., and et al. (1993). Cholinesterases as scavengers for organophosphorus compounds: protection of primate performance against soman toxicity. *Chem. Biol. Interact.*, 87: 285-293.

Ehrlich, G., Ginzberg, D., Loewenstein, Y., Glick, D., Kerem, B., Ben-Ari, S., Zakut, H., and Soreq, H. (1994). Population diversity and distinct haplotype frequencies associated with

ACHE and BCHE genes of Israeli Jews from trans-Caucasian Georgia and from Europe. *Genomics*, 22: 288-295.

Erb, C., Troost, J., Kopf, S., Schmitt, U., Loffelholz, K., Soreq, H., and Klein, J. (2001). Compensatory mechanisms enhance hippocampal acetylcholine release in transgenic mice expressing human acetylcholinesterase. *J. Neurochem.*, 77: 638-646.

Esposito, P., Chandler, N., Kandere, K., Basu, S., Jacobson, S., Connolly, R., Tutor, D., and Theoharides, T. C. (2002). Corticotropin-releasing hormone and brain mast cells regulate blood-brain-barrier permeability induced by acute stress. *J. Pharmacol. Exp. Ther.*, 303: 1061-1066.

Esposito, P., Gheorghe, D., Kandere, K., Pang, X., Connolly, R., Jacobson, S., and Theoharides, T. C. (2001). Acute stress increases permeability of the blood-brain-barrier through activation of brain mast cells. *Brain Res.*, 888: 117-127.

Evans, W. E., and Relling, M. V. (1999). Pharmacogenomics: translating functional genomics into rational therapeutics. *Science*, 286: 487-491.

Farchi, N., Soreq, H., and Hochner, B. (in press). Chronic acetylcholinesterase overexpression induces multileveled aberrations in neuromuscular physiology. *J. Physiol.*

Fibiger, H. C. (1991). Cholinergic mechanisms in learning, memory and dementia: a review of recent evidence. *Trends Neurosci.*, 14: 220-223.

Friedman, A., Kaufer, D., Pavlovsky, L., and Soreq, H. (1998). Cholinergic excitation induces activity-dependent electrophysiological and transcriptional responses in hippocampal slices. *J. Physio. Paris*, 92: 329-335.

Friedman, A., Kaufer, D., Shemer, J., Hendler, I., Soreq, H., and Tur-Kaspa, I. (1996). Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. *Nat. Med.*, 2: 1382-1385.

Fullerton, C. S., and Ursano, R. J. (1990). Behavioral and psychological responses to chemical and biological warfare. *Mil. Med.*, 155: 54-59.

Furey, M. L., Pietrini, P., and Haxby, J. V. (2000). Cholinergic enhancement and increased selectivity of perceptual processing during working memory. *Science*, 290: 2315-2319.

Gheusi, G., Bluthe, R. M., Goodall, G., and Dantzer, R. (1994). Ethological study of the effects of tetrahydroaminoacridine (THA) on social recognition in rats. *Psychopharmacol. Berl.*, 114: 644-650.

Giacobini, E., Spiegel, R., Enz, A., Veroff, A. E., and Cutler, N. R. (2002). Inhibition of acetyl- and butyryl-cholinesterase in the cerebrospinal fluid of patients with Alzheimer's disease by rivastigmine: correlation with cognitive benefit. *J. Neural Transm.*, 109: 1053-1065.

Golomb, B. (1999). A Review of the Scientific Literature as it pertains to Gulf War Illnesses, Vol 2, Rand Corp. Santa Monica.

Grant, A. D., Shapira, M., and Soreq, H. (2001). Genomic dissection reveals locus response to stress for mammalian acetylcholinesterase. *Cell. Mol. Neurobiol.*, 21: 783-797.

Gray, R., Rajan, A. S., Radcliffe, K. A., Yakehiro, M., and Dani, J. A. (1996). Hippocampal synaptic transmission enhanced by low concentrations of nicotine. *Nature*, 383: 713-716.

Greenfield, S. (1996). Non-classical actions of cholinesterases: role in cellular differentiation, tumorigenesis and Alzheimer's disease. *Neurochem. Int.*, 28: 485-490.

Greenfield, S., and Vaux, D. J. (2002). Parkinson's disease, Alzheimer's disease and motor neurone disease: identifying a common mechanism. *Neuroscience*, 113: 485-492.

Grifman, M., Galyam, N., Seidman, S., and Soreq, H. (1998). Functional redundancy of acetylcholinesterase and neuroligin in mammalian neuritogenesis. *Proc. Natl. Acad. Sci. U. S. A.*, 95: 13935-13940.

Grisaru, D., Deutch, V., Shapira, M., Galyam, N., Lessing, B., Eldor, A., and Soreq, H. (2001). ARP, a peptide derived from the stress-associated acetylcholinesterase variant, has hematopoietic growth promoting activities. *Mol. Med.*, 7: 93-105.

Grisaru, D., Lev-Lehman, E., Shapira, M., Chaikin, E., Lessing, J. B., Eldor, A., Eckstein, F., and Soreq, H. (1999a). Human osteogenesis involves differentiation-dependent increases in the morphogenically active 3' alternative splicing variant of acetylcholinesterase. *Mol. Cell Biol.*, 19: 788-795.

Grisaru, D., Sternfeld, M., Eldor, A., Glick, D., and Soreq, H. (1999b). Structural roles of acetylcholinesterase variants in biology and pathology. *Eur. J. Biochem.*, 264: 672-686.

Hamos, J. E., Oblas, B., Pulaski-Salo, D., Welch, W. J., Bole, D. G., and Drachman, D. A. (1991). Expression of heat shock proteins in Alzheimer's disease. *Neurology*, 41: 345-350.

Hillegaart, V., and Ahlenius, S. (1994). Time course for synchronization of spontaneous locomotor activity in the rat following reversal of the daylight (12:12 h) cycle. *Physiol. Behav.*, 55: 73-75.

Imperato, A., Puglisi-Allegra, S., Casolini, P., and Angelucci, L. (1991). Changes in brain dopamine and acetylcholine release during and following stress are independent of the pituitary-adrenocortical axis. *Brain Res.*, 538: 111-117.

Inestrosa, N. C., Alvarez, A., Godoy, J., Reyes, A., and De Ferrari, G. V. (2000). Acetylcholinesterase-amyloid-beta-peptide interaction and Wnt signaling involvement in Abeta neurotoxicity. *Acta Neurol. Scand.*, Suppl. 176: 53-59.

Inestrosa, N. C., Alvarez, A., Perez, C. A., Moreno, R. D., Vicente, M., Linker, C., Casanueva, O. I., Soto, C., and Garrido, J. (1996). Acetylcholinesterase accelerates assembly of amyloid-ß peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. *Neuron*, 16: 881-891.

Kalow, W., and Genest, K. (1957). A method for detection of atypical forms of human serum cholinesterase: Determination of dibucaine numbers. *Can. J. Biochem.*, 35: 339-346.

Kaneto, H. (1997). Learning/memory processes under stress conditions. *Behav. Brain Res.*, 83: 71-74.

Kant, G. J., Bauman, R. A., Feaster, S. R., Anderson, S. M., Saviolakis, G. A., and Garcia, G. E. (2001). The combined effects of pyridostigmine and chronic stress on brain cortical and blood acetylcholinesterase, corticosterone, prolactin and alternation performance in rats. *Pharmacol. Biochem. Behav.*, 70: 209-218.

Karpel, R., Ben Aziz Aloya, R., Sternfeld, M., Ehrlich, G., Ginzberg, D., Tarroni, P., Clementi, F., Zakut, H., and Soreq, H. (1994). Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. *Exp. Cell Res.*, 210: 268-277.

Karpel, R., Sternfeld, M., Ginzberg, D., Guhl, E., Graessmann, A., and Soreq, H. (1996). Overexpression of alternative human acetylcholinesterase forms modulates process extensions in cultured glioma cells. *J. Neurochem.*, 66: 114-123.

Kaufer, D., Friedman, A., Seidman, S., and Soreq, H. (1998). Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature*, 393: 373-377.

Kaufer, D., Friedman, A., Seidman, S., and Soreq, H. (1999). Anticholinesterases induce multigenic transcriptional feedback response suppressing cholinergic neurotransmission. *Chem. Biol. Interact.*, 119-120: 349-360.

Kirschbaum, C., Wolf, O. T., May, M., Wippich, W., and Hellhammer, D. H. (1996). Stress- and treatment-induced elevations of cortisol levels associated with impaired declarative memory in healthy adults. *Life Sci.*, 58: 1475-1483.

Koenigsberger, C., Chiappa, S., and Brimijoin, S. (1997). Neurite differentiation is modulated in neuroblastoma cells engineered for altered acetylcholinesterase expression. *J. Neurochem.*, 69: 1389-1397.

Kronman, C., Chitlaru, T., Elhanany, E., Velan, B., and Shafferman, A. (2000). Hierarchy of post-translation modifications involved in the circulatory longevity of glycoproteins. Demonstration of concerted contributions of glycan sialylation and subunit assembly to the pharmacokinetic behavior of acetylcholinesterase. *J. Biol. Chem.*, 275: 29488-29502.

Kubo, S., Kitamura, O., Orihara, Y., Ogata, M., Tokunaga, I., and Nakasono, I. (1998). Immunohistochemical diagnosis and significance of forensic neuropathological changes. *J. Med. Invest.*, 44: 109-119.

La Du, B. N., Bartels, C. F., Nogueira, C. P., Hajra, A., Lightstone, H., Van der Spek, A., and Lockridge, O. (1990). Phenotypic and molecular biological analysis of human butyrylcholinesterase variants. *Clin. Biochem.*, 23: 423-431.

Layer, P. G. (1996). Non-classical actions of cholinesterases: role in cellular differentiation, tumorigenesis and Alzheimer's disease. *Neurochem. Int.*, 28: 491-495.

Lehmann, D. J., Johnston, C., and Smith, A. D. (1997). Synergy between the genes for butyrylcholinesterase K variant and apolipoprotein E4 in late-onset confirmed Alzheimer's disease. *Hum. Mol. Genet.*, 6: 1933-1936.

Lehmann, D. J., Nagy, Z., Litchfield, S., Borja, M. C., and Smith, A. D. (2000). Association of butyrylcholinesterase K variant with cholinesterase-positive neuritic plaques in the temporal cortex in late-onset Alzheimer's disease. *Hum. Genet.*, 106: 447-452.

Levene, C., Bar-Shany, S., Manny, N., Moulds, J. J., and Cohen, T. (1987). The Yt blood groups in Israeli Jews, Arabs, and Druse. *Transfusion*, 27: 471-474.

Li, R., Fujitani, N., Jia, J. T., and Kimura, H. (1998). Immunohistochemical indicators of early brain injury: an experimental study using the fluid-percussion model in cats. *Am. J. Forensic Med. Pathol.*, 19: 129-136.

Li, Y., Liu, L., Kang, J., Sheng, J. G., Barger, S. W., Mrak, R. E., and Griffin, W. S. (2000). Neuronal-glial interactions mediated by interleukin-1 enhance neuronal acetylcholinesterase activity and mRNA expression. *J. Neurosci.*, 20: 149-155.

Lightman, S. L., Windle, R. J., Julian, M. D., Harbuz, M. S., Shanks, N., Wood, S. A., Kershaw, Y. M., and Ingram, C. D. (2000). Significance of pulsatility in the HPA axis. *Novartis Found. Symp.*, 227: 244-257; discussion 257-260.

Liu, W. F. (1992). Acute effects of oral low doses of pyridostigmine on simple visual discrimination and unconditioned consummatory acts in rats. *Pharmacol. Biochem. Behav.*, 41: 251-254.

Llinas, R. R., and Greenfield, S. A. (1987). On-line visualization of dendritic release of acetylcholinesterase from mammalian substantia nigra neurons. *Proc. Natl. Acad. Sci. U. S. A.*, 84: 3047-3050.

Luo, Z. D., Wang, Y., Werlen, G., Camp, S., Chien, K. R., and Taylor, P. (1999). Calcineurin enhances acetylcholinesterase mRNA stability during C2-C12 muscle cell differentiation. *Mol. Pharmacol.*, 56: 886-894.

Lupien, S. J., and Lepage, M. (2001). Stress, memory, and the hippocampus: can't live with it, can't live without it. *Behav. Brain Res.*, 127: 137-158.

Lupien, S. J., Maheu, F., Tu, M. T., Lemay, M., McEwen, B. S., and Meaney, M. J. (1999). Increased cortisol levels and impaired cognition in human aging: implication for depression and dementia in later life. *Rev. Neurosci.*, 10: 117-139.

Luth, H. J., Holzer, M., Gartner, U., Staufenbiel, M., and Arendt, T. (2001). Expression of endothelial and inducible NOS-isoforms is increased in Alzheimer's disease, in APP23 transgenic mice and after experimental brain lesion in rat: evidence for an induction by amyloid pathology. *Brain Res.*, 913: 57-67.

Maes, M., Song, C., Lin, A., De Jongh, R., Van Gastel, A., Kenis, G., Bosmans, E., De Meester, I., Benoy, I., Neels, H., et al. (1998). The effects of psychological stress on humans: increased production of pro-inflammatory cytokines and a Th1-like response in stress-induced anxiety. Cytokine, 10: 313-318.

Marchot, P., Prowse, C. N., Kanter, J., Camp, S., Ackermann, E. J., Radic, Z., Bougis, P. E., and Taylor, P. (1997). Expression and activity of mutants of fasciculin, a peptidic acetylcholinesterase inhibitor from mamba venom. *J. Biol. Chem.*, 272: 3502-3510.

Marx, J. (1995). How the glucocorticoids suppress immunity. Science 270, 232.

Massoulie, J. (2000). Molecular forms and anchoring of acetylcholinesterase. In E. Giacobini (Ed.), *Cholinesterases and Cholinesterase Inhibitors*, Martin Dunitz, London, pp. 81-101.

McEwen, B. S. (1999). Stress and hippocampal plasticity. *Annu. Rev. Neurosci.*, 22: 105-122.

Meshorer, E., Erb, C., Gazit, R., Pavlovsky, L., Kaufer, D., Friedman, A., Glick, D., Ben-Arie, N., and Soreq, H. (2002). Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. *Science*, 295: 508-512.

Mesulam, M., Carson, K., Price, B., and Geula, C. (1992). Cholinesterases in the amyloid angiopathy of Alzheimer's disease. Ann. Neurol., 31: 565-569.

Mesulam, M. M., Guillozet, A., Shaw, P., Levey, A., Duysen, E. G., and Lockridge, O. (2002). Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. *Neuroscience*, 110: 627-639.

Millard, C. B., and Broomfield, C. A. (1995). Anticholinesterases: medical applications of neurochemical principles. *J. Neurochem.*, 64: 1909-1918.

Mor, I., Grisaru, D., Titelbaum, L., Evron, T., Richler, C., Wahrman, J., Sternfeld, M., Yogev, L., Meiri, N., Seidman, S., and Soreq, H. (2001). Modified testicular expression of stress-associated "readthrough" acetylcholiensterase predicts male infertility. *FASEB J.*, 15: 2039-2041.

Munck, A., and Naray-Fejes-Toth, A. (1994). Glucocorticoids and stress: permissive and suppressive actions. *Ann. N. Y. Acad. Sci.*, 746: 115-130; discussion 131-113.

Nguyen, K. T., Deak, T., Owens, S. M., Kohno, T., Fleshner, M., Watkins, L. R., and Maier, S. F. (1998). Exposure to acute stress induces brain interleukin-1beta protein in the rat. *J. Neurosci.*, 18: 2239-2246.

Nguyen, T., and Sudhof, T. C. (1997). Binding properties of neuroligin 1 and neurexin 1ß reveal function as heterophilic cell adhesion molecules. *J. Biol. Chem.*, 272: 26032-26039.

Nicole, S., Diaz, C. C., Frugier, T., and Melki, J. (2002). Spinal muscular atrophy: recent advances and future prospects. *Muscle Nerve*, 26: 4-13.

Nitsch, R. M., Rossner, S., Albrecht, C., Mayhaus, M., Enderich, J., Schliebs, R., Wegner, M., Arendt, T., and von der Kammer, H. (1998). Muscarinic acetylcholine receptors activate the acetylcholinesterase gene promoter. *J. Physiol. Paris*, 92: 257-264.

Ohno, K., Engel, A. G., Brengman, J. M., Shen, X. M., Heidenreich, F., Vincent, A., Milone, M., Tan, E., Demirci, M., Walsh, P., et al. (2000). The spectrum of mutations causing end-plate acetylcholinesterase deficiency. *Ann. Neurol.*, 47: 162-170.

Orr, R. M. (2001). Technology evaluation: fomivirsen, Isis Pharmaceuticals Inc/CIBA vision. *Curr. Opin. Mol. Ther.*, 3: 288-294.

Pakaski, M., and Kasa, P. (1992). Glial cells in coculture can increase the acetylcholinesterase activity in human brain endothelial cells. *Neurochem. Int.*, 21: 129-133.

Palmer, A. M. (2002). Pharmacotherapy for Alzheimer's disease: progress and prospects. *Trends Pharmacol. Sci.*, 23: 426-433.

Perrier, A. L., Massoulie, J., and Krejci, E. (2002). PRiMA: the membrane anchor of acetylcholinesterase in the brain. *Neuron*, 33: 275-285.

Perry, C., Sklan, E., Birikh, K., Shapira, M., Trejo, L., Eldor, A., and Soreq, H. (in press). Complex regulation of acetylcholinesterase gene expression in human brain tumors. *Oncogene*.

Perry, E., Martin-Ruiz, C., Lee, M., Griffiths, M., Johnson, M., Piggott, M., Haroutunian, V., Buxbaum, J. D., Nasland, J., Davis, K., et al. (2000). Nicotinic receptor subtypes in human brain ageing, Alzheimer and Lewy body diseases. *Eur. J. Pharmacol.*, 393: 215-222.

Ponting, C., Phillips, C., Davies, K., and Blake, D. (1997). PDZ domains: targeting signalling molecules to sub-membranous sites. *Bioessays*, 19: 469-479.

Raveh, L., Ashani, Y., Levy, D., De La Hoz, D., Wolfe, A. D., and Doctor, B. P. (1989). Acetylcholinesterase prophylaxis against organophosphate poisoning. Quantitative correlation between protection and blood-enzyme level in mice. *Biochem. Pharmacol.*, 38: 529-534.

Razon, N., Soreq, H., Roth, E., Bartal, A., and Silman, I. (1984). Characterization of activities and forms of cholinesterases in human primary brain tumors. **Exp. Neurol.**, 84,:681-695.

Rees, T., Hammond, P. I., Soreq, H., Younkin, S., and Brimijoin, S. (in press). Acetylcholinesterase promotes beta-amyloid plaques in cerebral cortex. *Neurobiol. Aging*.

Rehman, H. U., and Masson, E. A. (2001). Neuroendocrinology of ageing. *Age Ageing*, 30: 279-287.

Reichenberg, A., Yirmiya, R., Schuld, A., Kraus, T., Haack, M., Morag, A., and Pollmacher, T. (2001). Cytokine-associated emotional and cognitive disturbances in humans. *Arch. Gen. Psychiatry*, 58: 445-452.

Relyea, R. A., and Mills, N. (2001). Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (Hyla versicolor). *Proc. Natl. Acad. Sci. U. S. A.*, 98: 2491-2496.

Rosenstock, L., Keifer, M., Daniell, W., McConnell, R., and Claypoole, K. (1991). Chronic central nervous system effects of acute organophosphate pesticide intoxication. *Lancet*, 338: 223-227.

Rubin, L. L., and Staddon, J. M. (1999). The cell biology of the blood-brain barrier. *Annu. Rev. Neurosci.*, 22: 11-28.

Ruppin, E., and Reggia, J. A. (1995). Patterns of functional damage in neural network models of associative memory. *Neural Compu.*, 7: 1105-1127.

Sago, H., Carlson, E. J., Smith, D. J., Kilbridge, J., Rubin, E. M., Mobley, W. C., Epstein, C. J., and Huang, T. T. (1998). Ts1Cje, a partial trisomy 16 mouse model for Down syndrome, exhibits learning and behavioral abnormalities. *Proc. Natl. Acad. Sci. U. S. A.*, 95: 6256-6261.

Sberna, G., Saez Valero, J., Beyreuther, K., Masters, C. L., and Small, D. H. (1997). The amyloid ß-protein of Alzheimer's disease increases acetylcholinesterase expression by increasing intracellular calcium in embryonal carcinoma P19 cells. *J. Neurochem.*, 69: 1177-1184.

Scheiffele, P., Fan, J., Choih, J., Fetter, R., and Serafini, T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell*, 101: 657-669.

Selkoe, D. J. (2002). Alzheimer's disease is a synaptic failure. *Science*, 298: 789-791.

Servatius, R. J., Ottenweller, J. E., Beldowicz, D., Guo, W., Zhu, G., and Natelson, B. H. (1998). Persistently exaggerated startle responses in rats treated with pyridostigmine bromide. *J. Pharmacol. Exp. Ther.*, 287: 1020-1028.

Shapira, M., Grant, A., Korner, M., and Soreq, H. (2000a). Genomic and transcriptional characterization of the human ACHE locus: complex involvement with acquired and inherited diseases. *Isr. Med. Assoc. J.*, 2: 470-473.

Shapira, M., Tur-Kaspa, I., Bosgraaf, L., Livni, N., Grant, A. D., Grisaru, D., Korner, M., Ebstein, R. P., and Soreq, H. (2000b). A transcription-activating polymorphism in the ACHE promoter associated with acute sensitivity to anti-acetylcholinesterases. *Hum. Mol. Genet.*, 9: 1273-1281.

Shaw, N. (2002). The neurophysiology of concussion. Prog Neurobiol 67, 281.

Shih, T. M., and McDonough, J. H., Jr. (1997). Neurochemical mechanisms in soman-induced seizures. *J. Appl. Toxicol.*, 17: 255-264.

Shohami, E., Kaufer, D., Chen, Y., Seidman, S., Cohen, O., Ginzberg, D., Melamed-Book, N., Yirmiya, R., and Soreq, H. (2000). Antisense prevention of neuronal damages following head injury in mice. *J. Mol. Med.*, 78: 228-236.

Small, D. H., Michaelson, S., and Sberna, G. (1996). Non-classical actions of cholinesterases: role in cellular differentiation, tumorigenesis and Alzheimer's disease. *Neurochem. Int.*, 28: 453-483.

Somani, S. M., Husain, K., Asha, T., and Helfert, R. (2000). Interactive and delayed effects of pyridostigmine and physical stress on biochemical and histological changes in peripheral tissues of mice. *J. Appl. Toxicol.*, 20: 327-334.

Song, J. Y., Ichtchenko, K., Sudhof, T. C., and Brose, N. (1999). Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc. Natl. Acad. Sci. U. S. A.*, 96: 1100-1105.

Soreq, H., and Glick, D. (2000). Novel roles for cholinesterases in stress and inhibitor responses. In E. Giacobini (Ed.) *Cholinesterases and Cholinesterase Inhibitors: Basic, Preclinical and Clinical Aspects*, Martin Dunitz, London, pp. 47-61.

Spivak, B., Shohat, B., Mester, R., Avraham, S., Gil-Ad, I., Bleich, A., Valevski, A., and Weizman, A. (1997). Elevated levels of serum interleukin-1 beta in combat-related posttraumatic stress disorder. *Biol. Psychiatry*, 42: 345-348.

Stamm, S. (2002). Signals and their transduction pathways regulating alternative splicing: a new dimension of the human genome. *Hum. Mol. Genet.*,11: 2409-2416.

Stansbury, K., Haley, D., and Koeneker, A. (2000). Higher cortisol values facilitate spatial memory in toddlers. *Ann. N. Y. Acad. Sci.*, 911: 456-458.

Stephens, R., Spurgeon, A., Calvert, I. A., Beach, J., Levy, L. S., Berry, H., and Harrington, J. M. (1995). Neuropsychological effects of long-term exposure to organophosphates in sheep dip. *Lancet*, 345: 1135-1139.

Sternfeld, M., Ming, G., Song, H., Sela, K., Timberg, R., Poo, M., and Soreq, H. (1998a). Acetylcholinesterase enhances neurite growth and synapse development through alternative

contributions of its hydrolytic capacity, core protein, and variable C termini. *J. Neurosci.*, 18: 1240-1249.

Sternfeld, M., Patrick, J. D., and Soreq, H. (1998b). Position effect variegations and brain-specific silencing in transgenic mice overexpressing human acetylcholinesterase variants. *J. Physiol. Paris*, 92: 249-255.

Sternfeld, M., Shoham, S., Klein, O., Flores-Flores, C., Evron, T., Idelson, G. H., Kitsberg, D., Patrick, J. W., and Soreq, H. (2000). Excess "readthrough" acetylcholinesterase attenuates but the "synaptic" variant intensifies neurodeterioration correlates. *Proc. Natl. Acad. Sci. U. S. A* , 97: 8647-8652.

Stitcher, D. L., Harris, L. W., Heyl, W. C., and Alter, S. C. (1978). Effects of pyridostigmine and cholinolytics on cholinesterase and acetylcholine in Soman poisoned rats. *Drug Chem. Toxicol.*, 1: 355-362.

Taylor, P. (1996). Anticholinesterase agents. In J. G. Hardman, and L. E. Limbird (Eds.) *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, pp. 161-176.

Tokuyama, W., Okuno, H., Hashimoto, T., Xin Li, Y., and Miyashita, Y. (2000). BDNF upregulation during declarative memory formation in monkey inferior temporal cortex. *Nat. Neurosci.*, 3: 1134-1142.

Tracey, K. J., Czura, C. J., and Ivanova, S. (2001). Mind over immunity. *FASEB J.*, 15: 1575-1576.

Tsigelny, I., Shindyalov, I. N., Bourne, P. E., Sudhof, T. C., and Taylor, P. (2000). Common EF-hand motifs in cholinesterases and neuroligins suggest a role for Ca2+ binding in cell surface associations. *Protein Sci.*, 9: 180-185.

van Haaren, F., De Jongh, R., Hoy, J. B., Karlix, J. L., Schmidt, C. J., Tebbett, I. R., and Wielbo, D. (1999). The effects of acute and repeated pyridostigmine bromide administration on response acquisition with immediate and delayed reinforcement. *Pharmacol. Biochem. Behav.*, 62: 389-394.

Vereker, E., O'Donnell, E., and Lynch, M. A. (2000). The inhibitory effect of interleukin-1beta on long-term potentiation is coupled with increased activity of stress-activated protein kinases. *J. Neurosci.*, 20: 6811-6819.

Vincent, A., and Drachman, D. B. (2002). Myasthenia gravis. *Adv. Neurol.*, 88: 159-188.

Vyas, A., Mitra, R., Shankaranarayana Rao, B. S., and Chattarji, S. (2002). Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J. Neurosci.*, 22: 6810-6818.

Weeber, E. J., Atkins, C. M., Selcher, J. C., Varga, A. W., Mirnikjoo, B., Paylor, R., Leitges, M., and Sweatt, J. D. (2000). A role for the beta isoform of protein kinase C in fear conditioning. *J. Neurosci.*, 20: 5906-5914.

Weibel, L., Follenius, M., and Brandenberger, G. (1999). [Biologic rhythms: their changes in night-shift workers]. *Presse Med.*, 28: 252-258.

Wilckens, T., and De Rijk, R. (1997). Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunol. Today*, 18: 418-424.

Winslow, J. T., and Camacho, F. (1995). Cholinergic modulation of a decrement in social investigation following repeated contacts between mice. *Psychopharmacol. Berl.*, 121: 164-172.

Wolfe, A. D., Blick, D. W., Murphy, M. R., Miller, S. A., Gentry, M. K., Hartgraves, S. L., and Doctor, B. P. (1992). Use of cholinesterases as pretreatment drugs for the protection of rhesus monkeys against soman toxicity. *Toxicol. Appl. Pharmacol.*, 117: 189-193.

Wolthuis, O. L., Groen, B., Busker, R. W., and van Helden, H. P. (1995). Effects of low doses of cholinesterase inhibitors on behavioral performance of robot-tested marmosets. *Pharmacol. Biochem. Behav.*, 51: 443-456.

Xie, J., and Black, D. L. (2001). A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels. *Nature*, 410: 936-939.

Xie, J., and McCobb, D. P. (1998). Control of alternative splicing of potassium channels by stress hormones. *Science*, 280: 443-446.

Xu, L., Anwyl, R., and Rowan, M. J. (1997). Behavioural stress facilitates the induction of long-term depression in the hippocampus. *Nature*, 387: 497-500.

Yirmiya, R., Pollak, Y., Morag, M., Reichenberg, A., Barak, O., Avitsur, R., Shavit, Y., Ovadia, H., Weidenfeld, J., Morag, A., et al. (2000). Illness, cytokines, and depression. *Ann. N. Y. Acad. Sci.*, 917: 478-487.

Young, E., Cesena, T., Meiri, K. F., and Perrone-Bizzozero, N. I. (2002). Changes in protein kinase C (PKC) activity, isozyme translocation, and GAP-43 phosphorylation in the rat hippocampal formation after a single-trial contextual fear conditioning paradigm. *Hippocampus*, 12: 457-464.

Zakut, H., Even, L., Birkenfeld, S., Malinger, G., Zisling, R., and Soreq, H. (1988). Modified properties of serum cholinesterases in primary carcinomas. *Cancer*, 61: 727-737.

Zakut, H., Lieman Hurwitz, J., Zamir, R., Sindell, L., Ginzberg, D., and Soreq, H. (1991). Chorionic villus cDNA library displays expression of butyrylcholinesterase: putative genetic disposition for ecological danger. *Prenat. Diagn.* 11: 597-607.

Zenone, T., Bady, B., Souquet, P. J., and Bernard, J. P. (1992). [The Lambert-Eaton syndrome]. *Rev. Mal. Respir.*, 9: 483-490.

Zigmond, M. J. (1997). Do compensatory processes underlie the preclinical phase of neurodegenerative disease? Insights from an animal model of parkinsonism. *Neurobiol. Dis.*, 4: 247-253.

EXHIBIT C

25. Anderson, J. C., Douglas, R. J., Martin, K. A. C. & Nelson, J. C. Map of the synapses formed with the dendrites of spiny stellate neurons of cat visual cortex. J. Comp. Neurol. 341, 16–24 (1994).
26. Bringuier, V., Fregnac, Y., Baranyi, A., Debanne, D. & Shulz, D. Synaptic origin and stimulus dependency of neuronal oscillatory activity in the primary visual cortex of the cat. J. Physiol. 500, 751–774 (1997).
27. Debanne, D., Shulz, D. & Fregnac, Y. Activity-dependent regulation of 'on' and 'off' responses in cat visual cortical receptive fields. J. Physiol. 508, 523–548 (1998).
28. Nelson, K. in Methods in Enzymologie Ion Channels (eds Rudy, B. & Iverson, L. E.) 123–131 (Academic, New York, 1992).
29. Orban, G. A. Neuronal Operations in the Visual Cortex (Springer, Berlin, Heidelberg, 1984).

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Acute stress facilitates long-lasting changes in cholinergic gene expression

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Acute traumatic stress may lead to post-traumatic stress disorder (PTSD)[1], which is characterized by delayed neuropsychiatric symptoms including depression, irritability, and impaired cognitive performance[2]. Curiously, inhibitors of the acetylcholine-hydrolysing enzyme acetylcholinesterase may induce psychopathologies that are reminiscent of PTSD[3,4]. It is unknown how a single stressful event mediates long-term neuronal plasticity. Moreover, no mechanism has been proposed to explain the convergent neuropsychological outcomes of stress and of acetylcholinesterase inhibition. However, acute stress elicits a transient increase in the amounts released of the neurotransmitter acetylcholine and a phase of enhanced neuronal excitability[5]. Inhibitors of acetylcholinesterase also promote enhanced electrical brain activity[6], presumably by increasing the survival of acetylcholine at the synapse. Here we report that there is similar bidirectional modulation of genes that regulate acetylcholine availability after stress and blockade of acetylcholinesterase. These calcium-dependent changes in gene expression coincide with phases of rapid enhancement and delayed depression of neuronal excitability. Both of these phases are mediated by muscarinic acetylcholine receptors. Our results suggest a model in which robust cholinergic stimulation triggers rapid induction of the gene encoding the transcription factor c-Fos. This protein then mediates selective regulatory effects on the long-lasting activities of genes involved in acetylcholine metabolism.

The molecular mechanisms translating a traumatic life experience into long-term neuropsychological sequelae are expected to involve complex changes in gene regulation. We have previously shown that adult FVB/N mice subjected to either forced swimming stress or inhibitors of the acetylcholine-hydrolysing enzyme acetylcholinesterase (AChE) exhibit dramatic increases in levels of messenger RNA encoding the early immediate transcription factor c-Fos in the brain[7]. *In vitro*, sagittal corticohippocampal brain slices exposed to AChE inhibitors showed enhanced neuronal excitability and similar increases in cortical *c-fos* gene expression within 10 min (Fig. 1a). These increases are mediated by cholinergic stimulation of muscarinic acetylcholine receptors.

The presence of c-Fos-binding sites in the promoters of key cholinergic genes, such as the genes encoding AChE[8,9], the acetylcholine-synthesizing enzyme choline acetyltransferase (ChAT)[10], and the vesicular acetylcholine transporter (VAChT)[11], indicated that elevated c-Fos levels might activate regulatory pathway(s) leading to long-term changes in the expression of proteins mediating brain cholinergic neurotransmission. We performed quantitative reverse transcription with polymerase chain reaction (RT-PCR) on cortical RNA extracted either from mice 10–90 min after forced swimming or from brain slices after exposure to the cholinesterase

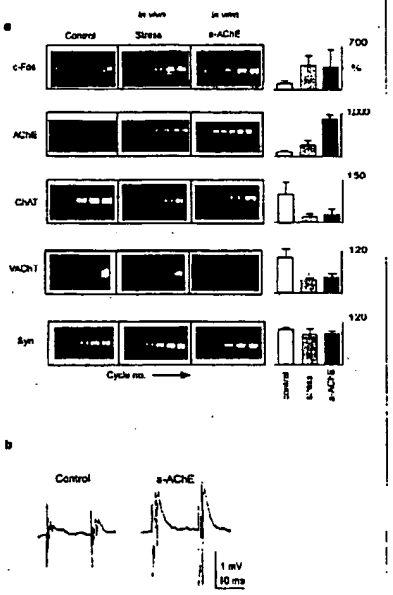

Figure 1 Acute stress and anticholinesterases modulate CNS gene expression similarly. a, RT-PCR analysis was performed on RNA extracted from the cortex of control mice and stressed mice or from sagittal corticohippocampal slices incubated with 1 μM DFP or 1mM pyridostigmine (s-AChI). Products were sampled every third cycle, electrophoresed, and stained with ethidium bromide. Data reflect c-Fos mRNA levels 10 min after stress or AChE inhibition, and AChE, ChAT, VAChT and synaptophysin (Syn) RNA levels 30 min after treatment. The figure shows representative gels and relative band intensities (mean ± s.d.), calculated from densitometric analysis of a single cycle verified to be within the linear range of product accumulation for the specific PCR reaction. On average, five RNA samples were analysed for each treatment group. For c-Fos, AChE, ChAT and VAChT, the differences in RNA levels observed between the control and either stress or s-AChE samples were all found to be statistically significant ($P < 0.02$) in a two-tailed Student's *t*-test. RNA from non-treated control animals generated patterns similar to those from non-treated slices (not shown). b, AChE inhibition increases neuronal excitability. The figure shows extracellular evoked potentials recorded in the CA1 area before (control) or 30 min following (s-AChI) addition of 1μM DFP to the perfusing solution. One of five reproducible experiments.

inhibitors diisopropylfluorophosphonate (DFP) or pyridostigmine. In all cases, AChE mRNA levels were markedly increased, whereas the levels of ChAT and VAChT mRNAs were reduced (Fig. 1a). These changes in gene expression lagged behind the increase in c-Fos levels by 20 min (Fig. 1a, and data not shown), consistent with the idea that c-Fos may activate or suppress cholinergic gene expression. The levels of mRNAs encoding synaptophysin, the L-type Ca^{2+} channel or glyceraldehyde phosphodehydrogenase remained unchanged (Fig. 1a, and data not shown). These results indicate that acute cholinergic stimulation promotes selective bidirectional changes in the expression of genes regulating acetylcholine metabolism. The combined effects of these changes should reduce the bioavailability of acetylcholine through suppressed synthesis/packaging and enhanced hydrolysis. It has been reported that transient increases and delayed reductions in acetylcholine levels accompany stress[5]. As modulated gene expression occurs *in vitro*, independently of the pituitary–adrenocortical axis, local mechanisms in the central nervous system are apparently enough to mediate this response.

Delayed reduction in acetylcholine levels after c-Fos induction predicts a secondary phase of suppressed neuronal excitability following both stress and AChE inhibition. To determine whether acetylcholinesterase inhibitors mediate both acute and delayed phases of cholinergic activity, we recorded extracellular potentials in the cell-body layer of the CA1 region of hippocampal slices; these potentials were evoked by orthodromic stimulation of the CA2/CA3 region of the stratum oriens enriched with cholinergic fibers[12]. Exposure to various AChE inhibitors prompted, within 1 h, increased population spike amplitude, rate of rise, and duration of paired-pulse facilitation under several stimulus intensities (Fig. 1b). When we extended AChE inhibition to 3 h, the augmented synaptic response and the population spikes were significantly muted, approaching responses seen under control conditions (Fig. 2a, b). These results show that AChE inhibitors mediate a transient, early phase of enhanced excitability that is followed by a delayed phase of suppressed neuronal activity. The non-hydrolysable acetylcholine analogue carbamylcholine promoted a similar and dose-dependent increase in amplitude and rate of rise of evoked population spikes (Fig. 2c). We could reversibly block both phases of inhibitor-mediated responses by adding the muscarinic antagonist atropine to the perfusion solution during the early phase (Fig. 2d). This indicates that the late phase of depressed activity represents a delayed response to a previous phase of acute muscarinic stimulation.

The *c-fos* gene includes a Ca^{2+}-responsive element and elevated c-Fos levels are a marker of neuronal hyperexcitation[13]. We therefore proposed that neuronal activity and/or intracellular accumulation of calcium play a role in translating the transient phase of cholinergic hyperactivation into changes in gene expression. The intracellular calcium chelator 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester (BAPTA AM) prevented enhanced paired-pulse facilitation in response to physostigmine (Fig. 3a), and both BAPTA-AM and the sodium-channel blocker tetrodotoxin (TTX) attenuated the changes in c-Fos and ChAT mRNA levels that are mediated by AChE inhibition (Fig. 3b). Our results indicate that intense cholinergic activation initiates a calcium- and neuronal-activity-dependent feedback

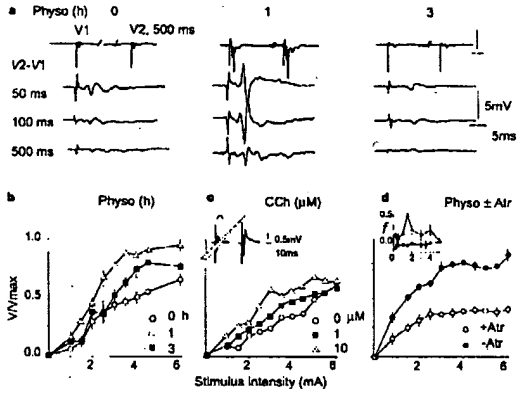

Figure 2. Delayed suppression of the hyperexcitation evoked by AChE inhibition. **a**, Enhancement of paired-pulse facilitation is transient. The figure shows the first and second evoked potentials (V_1, V_2) separated by a 500-ms interval or the difference ($V_2 - V_1$) at intervals 50, 100 or 500 ms for hippocampal slices under control conditions (0 h) or following 1 or 3 h of perfusion with the carbamate AChE inhibitor physostigmine (physo, 10 μM). **b**, Delayed suppression of increased population spike amplitudes. Average (± s.d.) relative amplitudes (V/V_{max}) of evoked population spikes under control conditions (open circles), or following 1 or 3 h of continuous perfusion of 10 μM physostigmine (physo) (grey triangles or filled squares, respectively), are shown. V_{max} for control is 1.04 mV at 9.5 mA. **c**, Carbachol increases population spike amplitudes. The electrophysiology is as in **b**. Control, empty circles; in presence of 1 μM carbachol (CCh), filled squares; 10 μM CCh, grey triangles. V_{max} for control is 1.98 mV at 8.5 mA. Inset shows the average of ten responses in control solution (left) or following 30-min perfusion with 10 μM CCh (right). **d**, Increased population spikes are mediated by muscarinic receptor activation. Results are shown from 1 h after coperfusion of physostigmine (10 μM) and atropine (Atr, 1 μM) (open circles) and 2 h later, following atropine washout (filled circles). The inset shows values of paired-pulse facilitation ($f = (V_2 - V_1)/V_1$) in the presence of physostigmine alone (triangles) as compared with physostigmine + atropine (circles). V_{max} for control is 2.25 mV at 7.5 mA.

pathway that works to suppress cholinergic neurotransmission through modulation of protein synthesis.

AChE activity in the neocortex and hippocampus, but not cerebellum, of animals exposed to a single stress session increased by two- to threefold within 50 min after stress and cortical activity remained significantly higher than that in control mice for over 80 h (Fig. 4; $P < 0.05$, two-tailed Student's t-test). The physiological relevance of this elevated brain AChE activity was seen as resistance to an anticholinesterase-induced drop in body temperature following stress. Intraperitoneal injection of pyridostigmine (0.2 mg kg^{-1}) induced a 0.9 ± 0.3 °C reduction in rectal temperature within 30 min in control mice ($n = 5$); no such drop was seen in mice subjected to forced swimming 24 h before injection of pyridostigmine (0.0 ± 0.3 °C, $n = 5$, $P < 0.005$, two-tailed Student's t-test). This observation indicates occlusion of the inhibitor effect by the effects of stress, and strengthens the contention that stress and cholinergic activation act on the brain through a common pathway involving elevated levels of synaptic acetylcholine.

Non-denaturing polyacrylamide gel electrophoresis revealed new, quickly migrating AChE form(s) in the brains of stressed mice (Fig. 4, inset). The pattern of gel migration corresponded closely with that of secreted, monomeric recombinant read-through AChE produced in *Xenopus* oocytes (Fig. 4 inset)[14]. Although a minor mRNA species encoding read-through AChE was previously detected in brain and in several tumour cell lines[13], its protein product has never been unequivocally identified *in vivo*. Following both stress and exposure to AChE inhibitors, a pronounced increase was observed in levels of this unspliced mRNA species in which pseudo-intron 4 is retained in the mature transcript (Fig. 5a). In contrast, no changes were seen in either the transcript containing the alternative 3′ exon 6 and encoding the dominant synaptic form of the enzyme, or in the transcript carrying alternative exon 5 and encoding the haematopoietic form of AChE (Fig. 5a). Thus, acute neuronal excitation mediated not only enhanced transcription, but also modified alternative splicing from the AChE gene, leading to *de novo* synthesis of the unique read-through AChE isoform.

In situ hybridization revealed low levels of read-through AChE mRNA in neuronal somata of cortical layers 2 and 5 of control mice. In contrast, both somata and apical dendrites of neurons from all cortical layers were intensely labelled for AChE mRNA following exposure to pyridostigmine (Fig. 5b). However, an exon-6-specific probe revealed similar levels of exon 6 AChE mRNA in somata of neurons in layers 2 and 5 of the parietal cortex of both control and inhibitor treated mice (Fig. 5b, and data not shown). As otherwise non-AChE-expressing cells began producing large amounts of a secretable, non-synaptic form of AChE after acute cholinergic stimulation, non-cholinergic, non-catalytic activities could be attributed to read-through AChE in modulating long-term neuronal reorganization following cholinergic insults[10–16]. This idea is consistent with reported homologies between AChE and a growing family of neuronal proteins involved in cell–cell interactions that include cytoplasmic domains capable of transducing signals[14].

Our results indicate that modulated cholinergic gene expression acts to reduce available acetylcholine and depress cholinergic neurotransmission following stress. This molecular compensation would play a crucial role in short term quietening of brain activity following a traumatic experience, but could have potentially damaging long-term implications. We previously reported that prolonged overexpression of AChE in the central nervous system of transgenic mice promotes delayed cognitive and neuroanatomical pathologies[14–17]. Our present results therefore indicate a common mechanism for the delayed neuropsychiatric pathologies

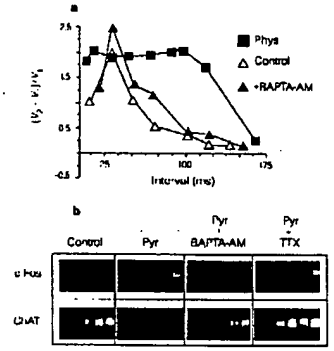

Figure 3 Physiological and transcriptional responses both depend on intracellular Ca^{2+} accumulation and Na^+ influx. a, Calcium chelator prevents enhancements. Paired-pulse facilitation was measured as in Fig. 2 in hippocampal slices under control conditions (open triangles), 1 h after the addition of 1 μM physostigmine (phys, filled squares) or 1 h after treatment with 1 μM physostigmine in the presence of the intracellular Ca^{2+} chelator BAPTA-AM (1 μM, filled triangles). BAPTA-AM alone had no effect on paired-pulse facilitation (data not shown). b, Suppression of the transcriptional response. c-Fos and ChAT mRNAs from control slices, or from slices treated for 1 h with 1 mM pyridostigmine (Pyr) alone or 1 mM Pyr with either BAPTA-AM (1 μM) or the Na^+-channel-blocker tetrodotoxin (TTX, 1 μM) were PCR amplified as in Fig. 1a.

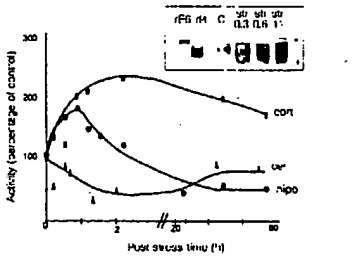

Figure 4 Long-term changes in AChE activity following stress. Average AChE activities (per cent of control) are shown in extracts of cortex (cort), cerebellum (cer), or hippocampus (hipp) from a total of 26 animals killed at the noted times after forced swimming. Specific activity (100%) in untreated controls was 3.98 ± 1.20, 1.95 ± 1.34, and 2.51 ± 0.30 nmol substrate hydrolysed per min per mg tissue for cortex, hippocampus and cerebellum, respectively. Inset shows that stress intensifies cortical AChE activity and diversifies its electrophoretic heterogeneity. Total proteins extracted from cortex of mice killed at the noted post stress (str) times were electrophoresed (70 μl per lane, 1 : 10 wt/vol) in 7% non-denaturing polyacrylamide gels; catalytically active AChE was stained histochemically[14]. Recombinant human exon 6/AChE (rE6) and read-through AChE (read-through intron 4, rI4) produced in *Xenopus* oocytes (1/5 oocytes per lane) served as known correlates. C, control.

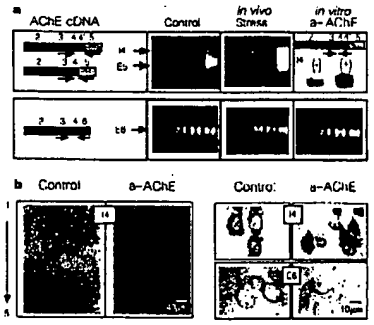

Figure 8 Selective induction of read-through AChE mRNA following stress and AChE inhibition. a, Kinetic follow-up of RT-PCR. Accumulated PCR products are derived from alternative AChE mRNAs under control, stress and anti-AChE (a-AChE) conditions. Positions of primer pairs specific for the read-through (intron 4, I4), synaptic (exon 6, E6) and erythrocyte (exon 5, E5) AChE mRNA subtypes are shown on the left. Endpoint products from a reaction using the I4-specific primer pair +1,381 to −I4 (74) were detected by hybridization with a radiolabelled riboprobe. b, *In situ* hybridization. Cortical layers 1–5 (low magnification, left) and representative pyramidal neurons from cortical layer 2 (high magnification, right) are shown before and after *in vivo* exposure to pyridostigmine (a-AChE, 2 mg kg^{-1}). Stress treatment induced similar changes (not shown).

associated with PTSD and those associated with anticholinesterases. Feedback pathways leading to raised AChE may also be associated with low-level exposure to common anticholinesterase drugs and insecticides. Moreover, our discovery of anticholinesterase-promoted feedback mechanisms may help to explain the limited efficacy of cholinesterase inhibitors in treating Alzheimer's disease[23,24] and the delayed neurocognitive disturbances reported by soldiers exposed to pyridostigmine or other anticholinesterases during the Gulf War[25,26]. □

Methods

Animal care and experimental protocols were carried out in accordance with institutional guidelines.

Forced swim stress protocol. This protocol was adapted for use in adult FVB/N mice as described[7]. Animals were subjected to two 4-min swim sessions in a water bath of 60 × 60 cm at 21 ± 1 °C.

Sagittal hippocampal brain slices. These slices were prepared and maintained as described[27], except that the concentration of KCl was 10 mM for molecular experiments and 5 mM for electrophysiological recordings. *Stratum oriens* fibres were stimulated with a bipolar tungsten electrode. Stimulation frequency was <0.3 Hz in all experiments. RT-PCR demonstrated the presence of stable mRNA for the key genes described for at least 12 h.

Kinetic follow-up of RT-PCR. This procedure was performed as described[7] using the following selective primer pairs. Numbers indicate nucleotide positions in the Genebank cDNA sequences. For the AChE gene, nucleotides +373 to −1,160 were used to detect a region common to all AChE mRNA subtypes; nucleotides +1,361 to 1,896 (exon 6) were used to detect mRNA encoding synaptic AChE; and nucleotides +1,361 to exon 5 (175) (intron 4/exon 5) were used to generate a 549 base pair (bp) product from read-through AChE mRNA and a 432 bp product from mRNA encoding the erythrocyte-linked form of the enzyme. For ChAT mRNA, nucleotides +83 to −646 were used; for c-Fos mRNA, nucleotides +1,604 to −1,306 were used; and for synaptophysin mRNA, nucleotides +212 to −660 were used.

***In situ* hybridization.** *In situ* hybridization was performed in 5 μm-thick paraffin-embedded cortical slices with 50-mer 5′-biotinylated, 2-*O*-methyl-protected complementary RNA probes[21] beginning at intron I4 position 74 or exon E6 position 1,392 of the mouse AChE gene. Probes were detected using an alkaline phosphatase/streptavidin conjugate and Fast Red (Boehringer) as a substrate. Counterstaining of nuclei was with Giemsa.

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1. Sapolsky, R. M. Why stress is bad for your brain. *Science* **273**, 749–750 (1996).
2. Gerrein, T. V. *et al.* Neurologic status of Vietnam veterans with chronic posttraumatic stress disorder. *J. Neuropsych. Clin. Neurosci.* **3**, 183–188 (1991).
3. Rosenbach, L., Keller, M., Connell, W. L., McConnell, R. & Chrysanthe, K. Chronic central nervous system effects of acute organophosphate pesticide intoxication. *Lancet* **338**, 223–227 (1991).
4. Wehling, L. The big easy serves up a feast to testing neuroscientists. *1st model for Gulf War syndrome? Science* **278**, 1406 (1996).
5. Imperato, A., Puglisi-Allegra, S., Casolini, P. & Angelucci, L. Changes in brain dopamine and acetylcholine release during and following stress are independent of the pituitary-adrenocortical axis. *Brain Res.* **538**, 111–117 (1991).
6. Fibiger, H. & Phillips, M. T. Basal activation of brain cortical acetylcholine release by systemic or intracortical microinjection of an irreversible acetylcholinesterase inhibitor: increased dialysate rate and inhibition of c fos. *Exp. Neurol.* **118**, 164–177 (1992).
7. Friedman, A. *et al.* Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. *Nature Med.* **2**, 1382–1385 (1996).
8. Bon Ame-Dabpay, K. *et al.* Expression of a human acetylcholinesterase promoter–reporter construct in developing neuromuscular junctions of *Xenopus embryos*. *Proc. Natl Acad. Sci. USA* **93**, 2471–2475 (1996).
9. Li, Y., Camp, S., Rachinsky, T. L., Bumgarner, C. & Taylor, P. Promoter elements and transcriptional control of the mouse acetylcholinesterase gene. *J. Biol. Chem.* **266**, 23613–23712 (1991).
10. Navaratnam, P. *et al.* Identification and analysis of the human choline acetyltransferase gene promoter. *Neuroreport* **6**, 2387–2390 (1995).
11. Cervini, R. *et al.* Specific molecular acetylcholine transporter promoters lie within the first intron of the rat choline acetyltransferase gene. *J. Biol. Chem.* **270**, 2663–2667 (1995).
12. Cole, A. E. & Nicoll, R. A. Acetylcholine mediates a slow synaptic potential in hippocampal pyramidal cells. *Science* **221**, 1299–1301 (1983).
13. Ghosh, A., Greer, C. A., Greenberg, M. E. Calcium regulation of gene expression in neuronal cells. *J. Neurobiol.* **25**, 294–303 (1994).
14. Seidman, S. *et al.* Synaptic and epidermal neuroadhesions of human acetylcholinesterase is encoded by alternative 3′-terminal exons. *Mol. Cell. Biol.* **15**, 3993–3982 (1995).
15. Koupal, K. *et al.* Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. *Exp. Cell Res.* **210**, 268–277 (1994).
16. Layer, P. G. & Willbold, E. Novel functions of cholinesterases in development, physiology and disease. *Prog. Histochem. Cytochem.* **29**, 1–99 (1995).
17. Koenigsberger, C., Chiappa, S. & Brimijoin, S. Neurite differentiation is modulated in reversible somatic cells engineered for altered acetylcholinesterase expression. *J. Neurochem.* **66**, 1309–1317 (1997).
18. Sternfeld, M. *et al.* Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein and variable C termini. *J. Neurosci.* **18**, 1240–1249 (1998).
19. Lichtenstein, E., Nguyen, T. & Sudhof, T. C. Structure, alternative splicing, and neurexin binding of multiple neuroligins. *J. Biol. Chem.* **272**, 2876–2882 (1996).
20. Beeri, R. *et al.* Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. *Curr. Biol.* **5**, 1063–1071 (1995).
21. Andres, C. *et al.* ACHE transgenic mice display embryonic modifications in spinal cord CHAT and neurexin 1β gene expression followed by late-onset neuromotor deterioration. *Proc. Natl Acad. Sci. USA* **94**, 8172–8178 (1997).

22. Beers, R. et al. Enhanced bromodeoxyuridine binding and attenuated dendritic branching in cognitively impaired ACHE-transgenic mice. J. Neurochem. 66, 2441–2451 (1997).
23. Knopp, M. J. et al. A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease. J. Am. Med. Assoc. 271, 985–991 (1994).
24. Wilcox, M. A. Tacrine for Alzheimer's disease: which patients, what dose? J. Am. Med. Assoc. 271, 1642–1648 (1994).
25. Shazeli, Y. et al. Survey of symptoms following uptake of pyridostigmine during the Persian gulf war. Isr. J. Med. Sci. 27, 656–658 (1991).
26. Milner, L. B. & Anstead, B. N., Pasquarosario, J. & Sallompas, M. Is there a Gulf War syndrome? J. Am. Med. Assoc. 275, 865 (1996).
27. Friedman, A. & Gutnick, M. J. Intracellular calcium and control of burst generation in neurons of guinea pig neocortex in vitro. Eur. J. Neurosci. 8, 371–381 (1996).

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Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A

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The NMDA (*N*-methyl-D-aspartate) subclass of glutamate receptor[1] is essential for the synaptic plasticity thought to underlie learning and memory[2–4] and for synaptic refinement during development[5,6]. It is currently believed that the NMDA receptor (NMDAR) is a heteromultimeric channel comprising the ubiquitous NR1 subunit and at least one regionally localized NR2 subunit[7–11]. Here we report the characterization of a regulatory NMDAR subunit, NR3A (formerly termed NMDAR-L or χ-1), which is expressed primarily during brain development[12,13]. NR3A co-immunoprecipitates with receptor subunits NR1 and NR2 in cerebrocortical extracts. In single-channel recordings from *Xenopus* oocytes, addition of NR3A to NR1 and NR2 leads to the appearance of a smaller unitary conductance. Genetic knockout of NR3A in mice results in enhanced NMDA responses and increased dendritic spines in early postnatal cerebrocortical neurons. These data suggest that NR3A is involved in the development of synaptic elements by modulating NMDAR activity.

We attempted to determine whether NR3A is a subunit of the NMDAR (Fig. 1a). First, using an anti-NR3A antibody, we defined the subcellular localization of NR3A in mouse cerebrocortical extracts at postnatal day 6 (P6). Like NR1, NR3A was selectively fractionated in a preparation enriched with postsynaptic densities (Fig. 1b). We next tested whether other glutamate-receptor subunits could be co-immunoprecipitated with NR3A from cerebrocortical extracts. NR1 and NR2B were detected in anti-NR3A immunoprecipitates, but non-NMDA glutamate receptor subunits GluR2, GluR6/7 and delta1/2 were absent (Fig. 1c, lane 3). These results suggest that NR3A is associated with NMDAR subunits in brain extracts, although they do not constitute direct evidence that NR3A is part of the NMDAR channel.

We performed single channel recordings from outside-out patches from *Xenopus* oocytes injected with NR1/NR2A ($n = 9$) or NR1/NR2A/NR3A ($n = 24$; Fig. 2). Our previous work had shown that expression of NR3A with NR1 and NR2 attenuated NMDA-evoked responses under two-electrode voltage clamp[12,13]. In our single-channel recordings, the presence of NR3A resulted in the appearance of a smaller unitary conductance (in 20 of 24 patches; Fig. 2b, d), in addition to the larger conductance previously observed for NR1/NR2A heteromers (Fig. 2a)[14]. Recordings were performed in low extracellular Ca^{2+} to minimize subconductance states. At −80 mV the larger state had a conductance of 75 ± 2.6 pS and the smaller 35 ± 3.4 pS (mean ± s.d. from amplitude histograms). Channels were analysed as described[15]. As the amount of injected NR3A cRNA was increased (ratio of

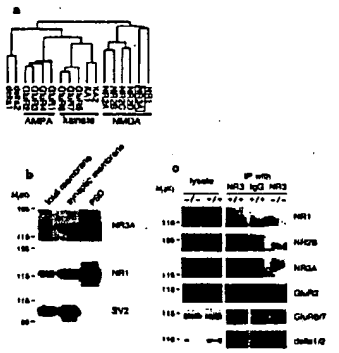

Figure 1 Biochemical characterization of NR3A. a, Alignment of members of the glutamate-receptor gene family based on amino-acid sequence homology. Progressive pairwise alignment of sequences[16] using the PILEUP program of the GCG software package for sequence analysis. Pharmacological classifications of subunits (see ref. 1) for functions of subunits other than NR3A) are shown under the alignment, and correlate well with groupings based on sequence similarities. The functional identification of NR3A as an NMDAR subunit extends this correlation. b, Subcellular localization of NR3A. Protein (25 μg) was applied to each lane of 8% SDS-PAGE, and immunoblotting was performed using antibodies against NR3A, NR1 and the presynaptic protein SV2. The 'total membrane' fraction contained membrane proteins from synapses, endoplasmic reticulum and Golgi apparatus. PSD, postsynaptic density fraction. The validity of our fractionation was confirmed by the distribution of the control proteins, NR1 and SV2. c, Co-immunoprecipitation of NR1 and NR2B with NR3A from P6 brain extracts. Lane 1, NR3A$^{-/-}$ extract; 2, wild-type extract; 3, immunoprecipitate (IP) with NR3A antibody from wild-type extract; 4, IP with rabbit anti-mouse IgG antibody from wild-type extract; and 5, IP with NR3A antibody from NR3A$^{-/-}$ extract. Protein (25 μg; lanes 1, 2) and IP from lysates initially containing 250 μg protein (lanes 3–5) were subjected to immunoblotting with the antibodies shown on the right. Control experiments (lanes 4, 5) validated the specificity of both the immunoprecipitation procedure and the NR3A antibody.

EXHIBIT D

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ORIGINAL ARTICLE

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Antisense prevention of neuronal damages following head injury in mice

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Abstract Closed head injury (CHI) is an important cause of death among young adults and a prominent risk factor for nonfamilial Alzheimer's disease. Emergency intervention following CHI should therefore strive to improve survival, promote recovery, and prevent delayed neuropathologies. We employed high-resolution nonradioactive in situ hybridization to determine whether a single intracerebroventricular injection of 500 ng 2′-O-methyl RNA-capped antisense oligonucleotide (AS-ODN) against acetylcholinesterase (AChE) mRNA blocks overexpression of the stress-related *readthrough* AChE (AChE-R) mRNA splicing variant in head-injured mice. Silver-based Golgi staining revealed pronounced dendrite outgrowth in somatosensory cortex of traumatized mice 14 days postinjury that was associated with sites of AChE-R mRNA overexpression and suppressed by anti-AChE AS-ODNs. Furthermore, antisense treatment reduced the number of dead CA3 hippocampal neurons in injured mice, and facilitated neurological recovery as determined by performance in tests of neuromotor coordination. In trauma-sensitive transgenic mice overproducing AChE, antisense treatment reduced mortality from 50% to 20%, similar to that displayed by head-injured control mice. These findings demonstrate the potential of antisense therapeutics in treating acute injury, and suggest antisense prevention of AChE-R overproduction to mitigate the detrimental consequences of various traumatic brain insults.

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Key words Antisense · Oligonucleotides · Head injury · Acetylcholinesterase · Emergency medicine

Abbreviations *AChE:* Acetylcholinesterase · *ARP:* Acetylcholinesterase *readthrough* peptide · *AS-ODN:* Antisense oligodeoxynucleotide · *CHI:* Closed head injury · *UTR:* Untranslated region

Introduction

Head trauma is an important cause of death among young adults [1, 2] and among the most significant risk factors known for nonfamilial Alzheimer's disease [3]. Emergency intervention in head injury must therefore consider not only short-term survival and recovery but also potential delayed neurological disorders. Robust production of stress hormones, transcription factors, cytokines, and neurotrophic factors have all been reported to occur in the injured brain [4, 5, 6]. Recently we demonstrated rapid and persistent overexpression of AChE-R in brain following acute psychological and chemical stressors associated with cholinergic excitation [7]. These observations suggested a role for AChE-R in stress responses accompanying various traumatic insults to the central nervous system [8]. Since the early post-CHI response phase includes a burst of released acetylcholine [9], we considered whether AChE-R acts as a stress response element following head injury. While acute stress responses likely promote survival, it has been argued that prolonged activation of stress-related genes initiates cascades of events with pleiotrophic and potentially deleterious effects [10]. Transgenic mice overexpressing human "synaptic" AChE-S in central cholinergic neurons exhibit delayed impairments in learning and memory [11] reminiscent of neuropathologies associated with posttraumatic stress disorder and anticholinesterase intoxication [8]. In this context, trauma-induced AChE-R might represent a candidate molecule contributing to the tight correlation between head injury and accelerated neurodeterioration.

Antisense oligonucleotides (AS-ODNs) eliciting selective destruction of specific messenger RNAs are ideally suited to the need for targeted downregulation of overexpressed proteins [12]. AS-ODNs are short, synthetic strands of DNA designed to hybridize with a specific target messenger RNA based on the rules of complementary Watson-Crick base pairing. Upon hybridization with AS-ODN, duplexed RNA becomes susceptible to RNase-mediated nucleolytic degradation, effectively blocking de novo synthesis of the unwanted protein. AS-ODNs have been used with remarkable success to modulate nervous system gene expression in the laboratory [13]. Nevertheless, restricted transport of oligonucleotides across the blood-brain barrier still poses a formidable obstacle to the application of antisense therapeutics to clinical neurology [14]. Here, we have used direct intracerebroventricular injections to deliver AS-ODNs to the injured brain, effectively suppressing stress-induced overexpression of AChE-R and improving the outcome of CHI.

Materials and methods

Closed head injury

Adult mice were anesthetized with ether to loss of pupillary and corneal reflexes. Closed head injury (CHI) was as described [15], in accordance with NIH guidelines for the use and care of laboratory animals and following approval by the Animal Care Committee of the Hebrew University of Jerusalem. Neurological functioning attesting to the severity of the injury was determined 1 h post-trauma, by recording of successful trials of crossing a 2- or 3-cm wide beam.

Antisense oligonucleotides

AS3 is a 20-mer oligodeoxynucleotide (5'-CTGCAATATTTTCT-TGCACC-3') complementary to a sequence in exon E2 of mouse AChEmRNA. ASB (5'-GACTTTGCTATGCAT3') is targeted to mRNA encoding mouse butyrylcholinesterase. In both cases, the last three 3' nucleotides were replaced with 2'-O-methyl ribonucleotide analogs. Oligonucleotides were synthesized, purified by high-pressure liquid chromatography and tested for purity by mass spectrometry at Hybridon (Rochester, N.Y., USA).

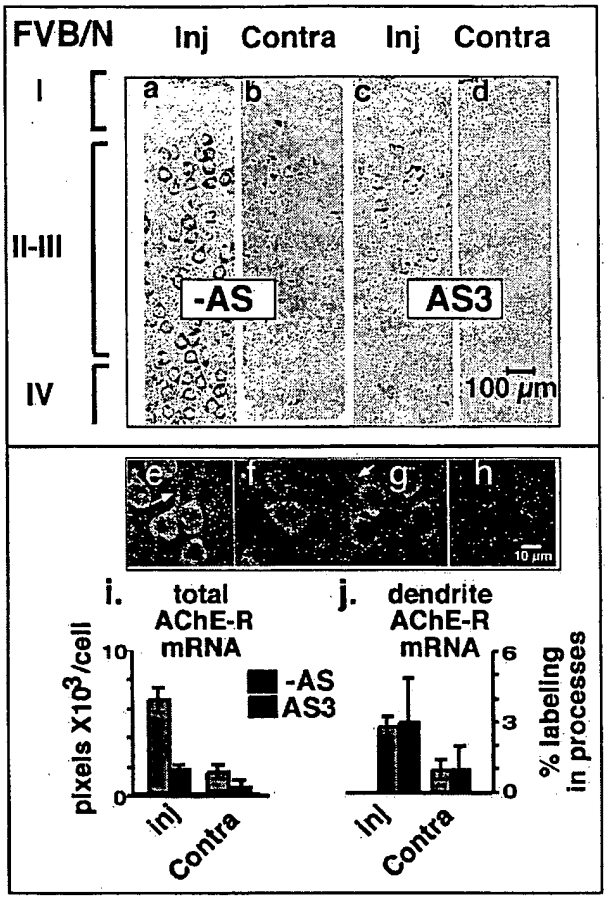
Golgi staining

Brains were fixed in 34 mM (1%) cobalt nitrate (18 h, room temperature), impregnated in 117 mM (2%) silver nitrate (24–48 h, room temperature) and processed in fresh Ramon y Cajal's developer. Paraffin sections were toned in 5 mM (0.2%) gold chloride, washed and counterstained with 2 mM alum carmine.

Results

To test whether AChE-R overproduction takes place under exposure to physical stress we exposed adult FVB/N mice to unilateral CHI [15] and used in situ hybridization to label AChE-R mRNA in brain sections. Consistent with previous findings, AChE-R mRNA was low in brains of uninjured mice ([7] and data not shown). In traumatized mice, however, intense labeling was observed in the contused hemisphere 14 days postinjury, especially close to the site of injury (Fig. 1a). Less prominent labeling was also evident in the contralateral hemisphere (Fig. 1b). Using a probe specific for AChE-S mRNA, we could not detect differences in the levels of this message between the injured and noninjured hemispheres (data not shown). To study the implications of AChE-R mRNA upregulation on the outcome of CHI, we employed AS-ODNs to selectively suppress postinjury AChE-R mRNA overproduction.

In cultured Saos-2 and PC12 cells low doses of AS3, a partially 2'-O-methyl-RNA modified antisense oligonucleotide targeted to the common domain in AChE mRNA, efficiently and selectively reduced AChE-R, but not AChE-S, mRNA levels ([16] and data not shown). Following CHI, a single intracerebroventricular injection of as little as 0.5 μg AS3 (70 pmol in a total volume of 10 μl) 1 h postinjury significantly prevented AChE-R mRNA accumulation in both brain hemispheres of injured mice (Fig. 1c, d). Treated mice displayed no signs of acute cholinergic hypofunction, suggesting that AChE-S levels were not significantly reduced. Confocal microscopy (Fig. 1 e-h) revealed four- to fivefold increased AChE-R mRNA labeling in both somata and apical dendrites of individual somatosensory cortical neurons in the contused versus contralateral hemisphere



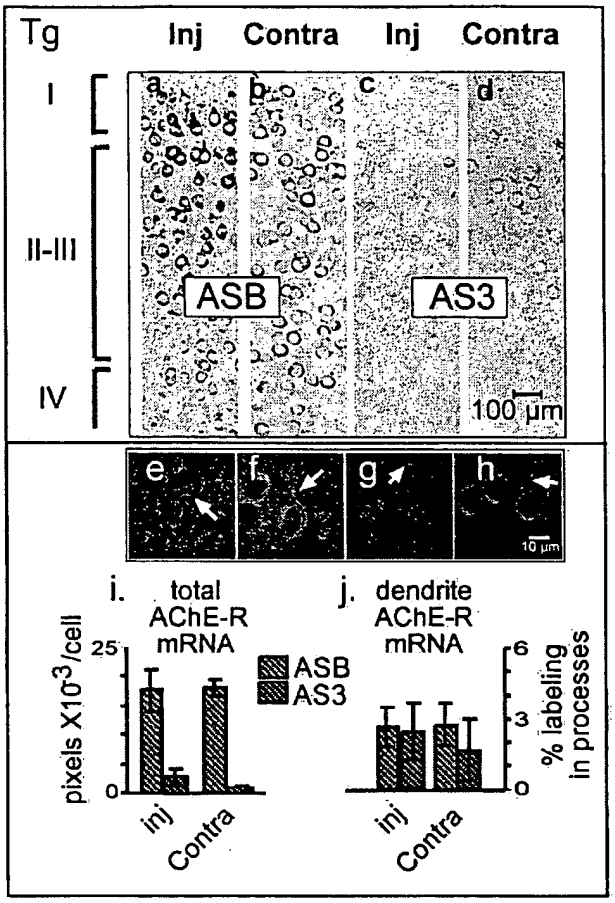


Fig. 1a–j Antisense oligonucleotides suppress trauma-induced accumulation of readthrough AChE-R mRNA in mouse brain. Male adult FVB/N mice were subjected to controlled closed head injury (left side of the head), injected after 1 h into the left ventricle with either saline or 500 ng of the partially 2-O-methyl-protected 20-mer AS-ODN AS3 and killed 14 days later. In situ hybridization was performed on 5-µm paraffin-embedded brain sections using a 50-mer biotinylated 2′-O-methyl cRNA probe targeted to intron I4 in mouse AChE-R mRNA. Staining was with Fast Red (Molecular Probes). **a–d** Brightfield digital photomicroscopy images of cortical layers I–IV from injured (**a**, **c**) and contralateral (**b**, **d**) hemispheres. *Red staining* marks sites of AChE-R mRNA accumulation. Note the intense staining of cortical neurons on the injured vs. contralateral side of the brain and the pronounced bilateral reduction in staining following a single unilateral injection of AS3. **e–h** Shown are representative confocal images of neurons from somatosensory cortex. Note the massive accumulation of AChE-R mRNA in both somata and apical dendrites (*white arrows*). AS3 dramatically suppressed the accumulation of AChE-R mRNA in neurons from both the injured (**e** vs. **g**) and contralateral (**f** vs. **h**) hemispheres and in both subcellular compartments. **i,j** Densitometric analysis was performed on confocal images of cortical neurons as depicted in **e–h**. **i** *Columns* Total number and standard deviation of pixels/cell for 10–20 neurons in each group (*left*) or the percentage of total pixels detected within dendrites (*right*) for the injured and contralateral hemispheres of mice injected with saline or AS3. Note the disproportionately high levels of AChE-R mRNA-specific fluorescence in neurons from the injured as compared to contralateral hemisphere, and the four- to sixfold reduced levels of staining in animals treated with AS3. The relative fraction of AChE-R mRNA appearing in dendrites was unmodified by antisense intervention (**j**)

Fig. 2a–j Head trauma elicits pronounced bilateral accumulation of endogenous AChE-R mRNA in AChE transgenic mice. Experimental details were as in Fig. 1 except that saline was replaced with an irrelevant oligonucleotide, ASB (see "Materials and methods"), as control. Note that transgenic mice, as opposed to control FVB/N mice display prominent overexpression of AChE-R mRNA in both the injured and noninjured brain hemispheres following head injury. **a–d** Brightfield microscopy. In brains from traumatized AChE transgenic mice with preexisting AChE excesses, similarly intense red hybridization signals were observed in cell bodies and dendrites of neurons from both the injured and contralateral hemispheres. AS3 effectively suppressed AChE-R mRNA expression in both hemispheres. **e–h** Confocal microscopy as in Fig. 1i–j. Densitometric analysis as in Fig. 1, note the more intense staining and higher dendrite localization of AChE-R mRNA in the contralateral hemisphere of transgenics as compared to FVB/N mice

(quantified in Fig. 1i). AS3 treatment conspicuously suppressed neuronal labeling in both subcellular compartments with approximately equal efficiency (Fig. 1j). Thus, CHI induced a persistent, at least 2-weeks-long overexpression of AChE-R mRNA in mouse brain, that was observed predominantly in the injured hemisphere. Moreover, AChE-R expression was prominently relieved by a single postinjury injection of an AS-ODN directed to a common exon in AChE mRNA.

To explore the molecular and cellular effects of preinjury AChE accumulation we traumatized transgenic mice

expressing up to twofold excesses of AChE-S in cholinergic CNS neurons. Following CHI, transgenic mice were injected with either AS3 or an antisense oligonucleotide targeted to mRNA encoding the nonrelevant, homologous butyrylcholinesterase (ASB; see above and [16]). Following in situ hybridization with an AChE-R mRNA specific probe, intense hybridization signals were observed in cortical neurons from both the injured and contralateral hemispheres of transgenic mice treated with the irrelevant oligonucleotide (Fig. 2a, b). In contrast, a single injection of AS3, effectively blocked AChE-R mRNA accumulation in both hemispheres (Fig. 2c, d). Confocal analysis demonstrated two- to fourfold increased levels of AChE-R mRNA in cells from either hemisphere of transgenic as compared with control mice (Fig. 2e-h). AS3 treatment reduced AChE-R mRNA to similarly low levels in both FVB/N and transgenics without affecting its subcellular distribution (Fig. 2i, j).

To test the effects of these AS-ODNs in noninjured transgenic mice we implanted 2-mm cannulae into the lateral ventricle. Ten days postimplantation we infused 25 ng ODN in a total volume of 1 µl twice at 24 h intervals. Mice were killed 24 h after the second injection and cortical homogenates prepared. An affinity-purified antibody raised against a recombinant fusion protein of glutathione transferase and the acetylcholinesterase *read-through* peptide (ARP), selective for AChE-R (Fig. 3a) labeled a ladder of bands in denaturing gels run with these homogenates. Based on densitometry of the six most prominent bands, we calculated that animals treated with ASB exhibited elevated levels (approximately 40% above naive mice) of immunoreactive polypeptides, especially the more rapidly migrating ones that likely reflect degradation products of AChE-R (Fig. 3b). This increase in AChE-R immunoreactivity was attributed to the trauma associated with surgical implantation of the cannula and was mostly suppressed by AS3 ($P \leq 0.01$; Fig. 3b). Immunolabeling with monoclonal antibodies targeted at the core domain failed to show suppression of immunoreactive protein by AS3 (data not shown). As these latter antibodies recognize both AChE-S and AChE-R, this analysis was consistent with the selectivity of AS3 effects on AChE-R mRNA. AS3-treated mice functioned well in behavioral tests of social recognition that are dependent on cholinergic function (data not shown), attesting to the integrity of normal cholinergic neurotransmission. Thus, the accumulation of AChE-R mRNA induced by even "mild" head injury appeared to be translated into elevated AChE-R protein levels. Moreover, AS3 blocked accumulation of both AChE-R mRNA and protein following trauma without promoting deleterious cognitive side effects in nonimpaired animals.

Regulated neurite outgrowth is likely an important component in short-term recovery from brain injury [17], but excessive neuronal sprouting following injury might be harmful and could promote posttraumatic epilepsy [18]. Since recent evidence attributes noncatalytic, neurite growth promoting activities to AChE [19], we con-

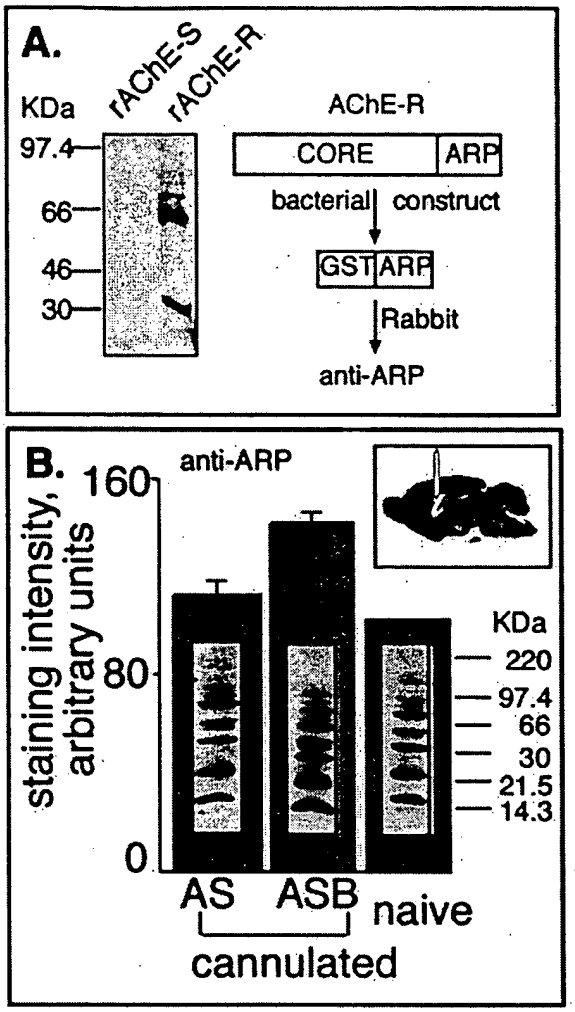


Fig. 3A,B AS3 suppresses trauma-induced overproduction of immunoreactive AChE-R protein. A Selective immunodetection of the AChE-R protein. To selectively label the AChE-R variant, rabbits were immunized with a recombinant fusion protein of glutathione transferase (*GST*) with the 26 amino acid C-terminal peptide unique to AChE-R (*ARP*). The resultant antiserum, affinity purified to remove anti-GST antibodies, selectively labeled recombinant (*r*) AChE-R produced in transfected Cos cells (66–67 K_d) and an apparent AChE-R degradation product (approx. 30 K_d), but not purified rAChE-S (Sigma, St. Louis, Mo., USA) in immunoblot analysis. **B** AS3 suppresses AChE-R labeling. Cortical homogenates from AChE transgenic mice injected icv with either AS3 or ASB on two consecutive days were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 4–20% reducing gel and probed with anti-ARP antiserum. Shown are the corresponding chemiluminescence-labeled lanes as *insets within a bar graph* representing the summation of densitometric analyses of the six most prominent bands in each lane. *Inset in right-hand corner* illustrates the position of the cannula. *Columns* represent mean±SEM for homogenates from six AS3- and four ASB-treated mice, respectively. A single representative control, untreated, nonoperated animal is shown (naive)

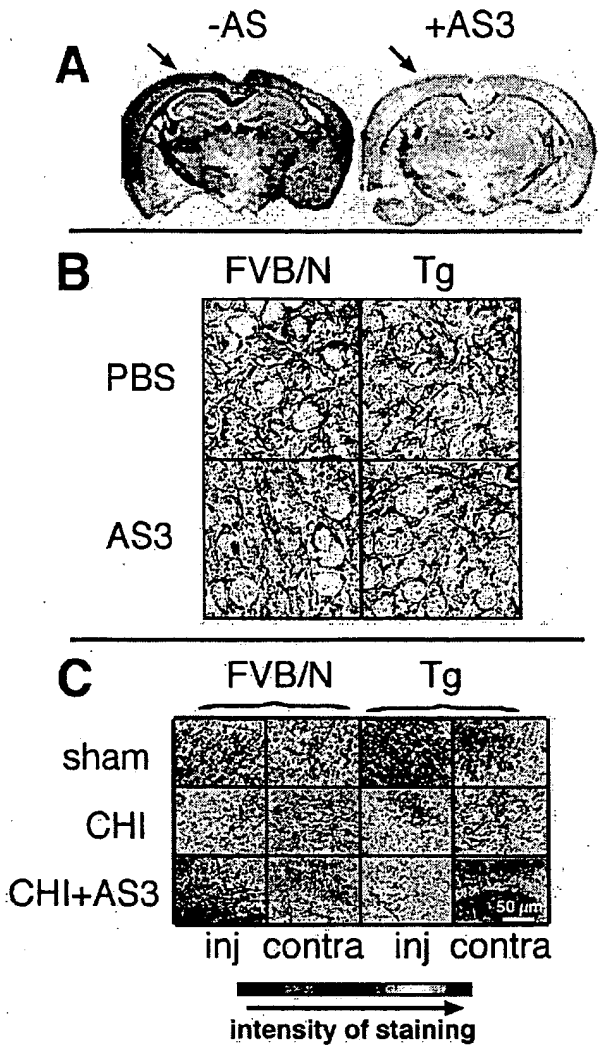


Fig. 4A–C Antisense treatment suppresses AChE-R mRNA and neurite growth and promotes neuron survival following head trauma. A Low magnification image of whole brain sections from head-injured FVB/N mice subjected to in situ hybridization as in Fig. 1A. Note that AChE-R mRNA is intensively expressed in the injured hemisphere (*left*), especially in the cortex, close to the site of injury (*arrows*). AS3 significantly suppressed AChE-R mRNA accumulation in both hemispheres. **B** Golgi staining was performed on brain sections from FVB/N or transgenic (*Tg*) mice subjected to CHI and injected intracerebroventricularly with either PBS or AS3. Shown are representative high magnification (×1000) images from layer IV of somatosensory cortex on the injured hemisphere 14 days following head injury. Note that AS3 treatment notably reduced the density of stained neurites in both FVB/N and transgenic mice. **C** Golgi staining performed on brain sections depicted in B was semiquantified in sections from uninjured (*sham*) FVB/N or AChE transgenic (*Tg*) mice and from mice untreated (*CHI*) or treated with AS3 (*CHI+AS3*) following closed head injury. Presented are pseudocolor representations of cortical sections in which stained neurites appear red. Note the intense Golgi staining, representing high neurite density in cortex from the injured hemisphere of FVB/N mice and the higher intensity in transgenic mice. Red signal was significantly reduced in both FVB/N and Tg mice treated with AS3

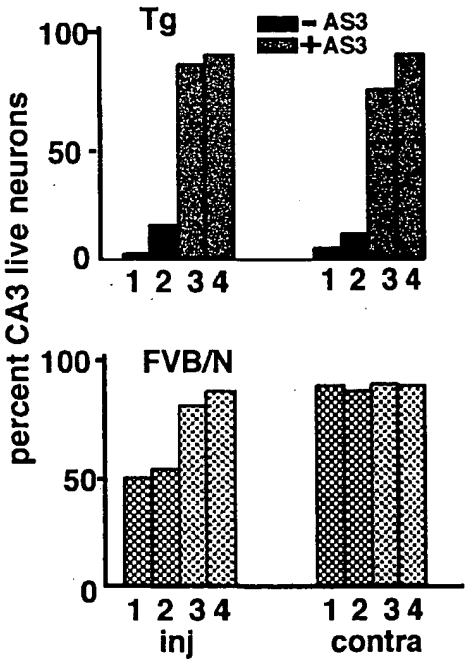


Fig. 5 Live and dead neurons in the CA3 hippocampal region were counted in two consecutive sections from four control and four transgenic mice 14 days following closed head injury with or without antisense treatment. Overt neuronal cell death was identified by the presence of pyknotic black cell bodies. For each mouse 150–200 cells were counted. *Columns* represent the percentage of live neurons counted for individual mice. Note the dramatic bilateral neuron loss suffered by transgenic as compared with FVB/N mice and the minimal neuron loss observed among antisense-treated animals

sidered the possibility that overexpressed AChE-R and/or its degradation products promote a phase of neurite outgrowth following CHI. To assess neuritic growth we performed quantitative image analysis on 100–200 μm² somatosensory cortical fields in Golgi-stained brain sections from mice 14 days posttrauma and searched for a correlation between neurite outgrowth and elevated AChE-R mRNA levels. Following head injury, pronounced increases in AChE-R mRNA labeling were colocalized with sites of intensified Golgi staining, especially in the somatosensory cortex around the site of injury, and particularly in transgenic mice (Fig. 4). AS3 significantly attenuated this increased staining in both FVB/N and transgenic mice, suggesting a correlation between AChE mRNA overproduction and induced neurite growth.

Excessive reinnervation following injury could subject hippocampal cells in the traumatized brain to hyperexcitation and glutamate toxicity. Indeed, hippocampal neurons, especially in the CA3 domain, are highly susceptible to cell death following brain injury [15, 20, 21]. Fourteen days following head injury we observed up to 50% loss of CA3 neurons in the contused hemisphere of FVB/N mice. In striking contrast, transgenic mice displayed up to 90% neuron death in CA3 of both hemi-

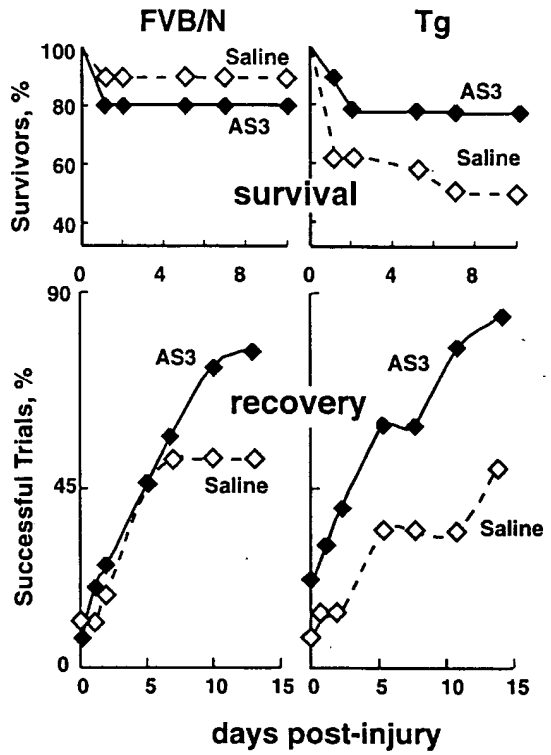


Fig. 6 Antisense treatment rescues survival and neurological recovery of injured mice. Survival and neurological recovery of FVB/N and AChE transgenic mice were monitored following CHI. One hour following trauma a neurological evaluation of the mice was taken using a graded balancing task of 2- and 3-cm-wide beam crossing. Recovery was monitored at various time points posttrauma for each mouse. Presented are percentages of surviving mice (*top*) and average percentage successes for surviving mice in the beam task (*bottom*) on the noted days following trauma. Note the high mortality and slow recovery of transgenic mice compared to controls and the improved outcome following a single AS3 treatment. Starting n=20–24 mice per group

spheres. Thus, overproduction of transgenic AChE appears to prime hippocampal neurons in CA3 for premature death following trauma. AS3 treatment afforded dramatic protection of hippocampal neurons in both control and transgenic animals subjected to CHI (Fig. 5).

The excessive loss of hippocampal neurons in brains of traumatized transgenic mice suggested that this strain would display a genetic vulnerability to CHI as compared to the parental FVB/N line. Indeed, young adult transgenic mice were considerably more vulnerable to unilateral CHI than age and sex-matched controls. Only 12 of 24 transgenics, as compared with 18 out of 20 FVB/N mice survived the first 10 days postinjury (Fig. 6). The 90% survival rate among FVB/N mice was similar to that displayed by other mouse strains subjected to this trauma protocol [15]. No further mortality was observed for the duration of the 30-day follow-up period. As overexpression of AChE was correlated with enhanced neurite outgrowth, the high mortality of AChE transgenic mice following CHI strengthens the idea that hyperinnervation may cause acute toxic effects during a phase of recovery lasting from 24 h up to 1 week posttrauma. Consistent with this conclusion, AS3 treatment retrieved the survival rate of AChE transgenics to 80%, comparable to that of FVB/N mice (Fig. 6).

Increased neuronal vulnerability to trauma implied that surviving transgenic mice would present impaired neurological recovery. To monitor neurological recovery we used a beam test, in which the percentage of mice that are capable of crossing 2- and 3-cm-wide beams is recorded. Success in this test is indicative of balanced and coordinated movement [15]. Although uninjured transgenic mice display some neuromotor impairments at the age of 4 months [22], transgenic and control mice scored very similarly 1 h postinjury (7 and 11%, respectively), indicating similar severity of trauma. Spontaneous recovery of transgenic survivors lagged significantly behind that of control mice throughout the 30 day follow-up period, especially during the first 10 days (Fig. 6 and data not shown). However, single dose AS3 treatment rapidly improved the performance of transgenic mice, to that of controls. Starting 7 days postinjury, AS3-treated FVB/N and transgenics both displayed improved performance as compared to untreated mice, both reaching ca. 80% of success, in comparison to 50% in the untreated groups (Fig. 6).

Discussion

We observed strong and persistent bilateral elevation in AChE-R mRNA levels and dramatic bilateral loss of hippocampal CA3 neurons in brains from AChE transgenic mice subjected to CHI. In comparison, trauma-induced overexpression of AChE-R and hippocampal cell loss in control FVB/N mice were primarily observed in the injured as opposed to contralateral hemispheres. Therefore, increased mortality among head-injured AChE transgenic mice and retarded neurological recovery of transgenic survivors associate robust, bilateral overexpression of AChE-R with poor prognosis following traumatic head injury. Despite progressive late-onset cognitive deficits, transgenic mice function normally until about 2 months of age [23]. This observation attests to the existence of compensatory mechanisms designed to overcome acetylcholine deficits imposed by overexpressed AChE ([24] and unpublished data). Since AChE-R expression is elevated by acute cholinergic stimulation [25], the pronounced bilateral overexpression of AChE-R in injured transgenic mice may therefore reflect heightened cholinergic activation in the noninjured hemisphere of these mice. As neurite growth is notably associated with increases in AChE activity (reviewed under [26]), it is understandable how antisense oligonucleotides blocking the initial postinjury accumulation of AChE-R could spare hippocampal neurons and minimize the morbidity of transgenic mice following CHI by reducing excessive neurite outgrowth. Prevention of this cascade of events also explains how a single, timely, and effective dose of

AS-ODN could provide long-lasting protection to the injured brain.

Head trauma patients may suffer delayed neurological deficits, including increased risk for Alzheimer's disease. The noncatalytic capacity of the AChE protein to induce β-amyloid aggregation [27] may be associated with this risk. The long-term neurological protection afforded by AS3 treatment thus remains to be tested in additional animal models, for example, mice that develop β-amyloid plaques [28]. Nevertheless, our findings provide the first experimental basis for the application of antisense technology to acute gene-oriented intervention in emergency medicine. In this light, AChE-R mRNA emerges as an appropriate target for such intervention where severe trauma to the nervous system is involved. Under psychological stress, AChE-R mRNA accumulation is preceded by an induction of c-fos [7]. Recently, Morrow and coworkers reported the use of AS-ODNs targeted towards c-fos mRNA to suppress central stress responses [29]. The higher ODN doses used in that study could reflect limited diffusion from the intracortical site of injection that was employed.

The increase in AChE-R and the accumulation of its degradation products in noninjured, cannulated transgenic mice most likely reflect surgery-related stress. Following two daily intracerebroventricular injections of 25 ng AS3, AChE-R immunolabeling was decreased to levels similar to those in the naive brain and significantly lower than that in brains of cannulated mice injected with the irrelevant ODN ASB. This experiment therefore provides a tentative time frame (48 h or less) for the AS3 effects, and demonstrates the potency of this ODN in blocking accumulation of AChE-R protein. In this context, it is important to note that AChE-R mRNA includes a 1094 basepair 3′ untranslated region (UTR) with high (66%) G, C content whereas AChE-S mRNA includes a considerably shorter, 219 basepair UTR. A long UTR predicts AChE-R mRNA to be more vulnerable to cellular nucleolytic degradation than AChE-S mRNA, consistent with the observation that AChE-R, but not AChE-S, mRNA is rapidly degraded in PC12 cells treated with actinomycin D [30]. The inherent instability of AChE-R mRNA could explain the preferential suppression of AChE-R over AChE-S mRNA by AS3 and AS1 [16], both of which target exon 2, common to all three alternative AChE mRNA splicing variants [31].

The AChE transcriptional response to stress involves modulation of the regular splicing pattern. It is not yet known whether the shift towards 3′ unspliced *read-through* AChE mRNA represents an active or passive process. However, stress-induced changes in alternative splicing have also been described for potassium channels, where stress hormones favor the splice variant encoding a channel subtype conferring repetitive firing characteristics [32]. Similarly, optic nerve injury induces altered splicing of the retinal NMDAR1 (NR1) receptor mRNA [33]. That active changes in cellular splicing machinery take place following stress is indicated by evidence for hypoxia-induced production of the YT521 splicing-related protein [34] and heat shock-induced splicing arrest [35]. Further studies are required to determine the mechanism(s) regulating stress-related changes in AChE mRNA splicing.

Following either stress or head injury, the subcellular distribution of AChE-R mRNA is altered, appearing also in the proximal domain of dendrites. The somatodendritic localization of this mRNA is not suppressed by AS3 and hence does not depend on mRNA content. Dendritic targeting of mRNAs encoding brain-derived neurotropic factor and TrkB has been reported in hippocampal neurons following activation of glutamate receptors [36]. These studies indicate the existence of neuronal activity dependent mechanisms for the subcellular distribution of neuronal mRNAs and suggest an association between mRNA redistribution and synaptic plasticity. The dendritic localization of AChE mRNA strengthens the notion that AChE contributes directly towards neurite growth in vivo, and suggests a role for this protein in dendrite abnormalities characteristic of the cognitively impaired AChE transgenic mice [11] and aged humans [8].

The prominent protection of hippocampal neurons, as well as improved rate of recovery with respect to regained neuromotor functions in head-injured AS3-treated FVB/N and transgenic mice, demonstrates the value of suppressing AChE-R levels for long-term recovery in terms of neurological functioning, and attributes profound implications for the potential application of antisense therapeutics to a clinical setting. That AChE-R mRNA accumulates under psychological [7], chemical [8], and physical (this report) stressors suggests that this transcript represents a general nervous system stress-response element. In view of our current findings, the grave consequences of AChE-R accumulation in the mammalian brain may extend to a wide range of maladies, including not only Alzheimer's disease but also poststroke depression [37], multiple sclerosis and amyotrophic lateral sclerosis. Moreover, our findings indicate a potentially increased risk for CHI victims with preinjury overexpression of brain AChE (for example, posttraumatic stress patients).

The side effects of repetitive administration of AS-ODNs in the nervous system have not been thoroughly studied, but are not expected to be severe [12, 13, 14]. Indeed, the recent United States Food and Drug Administration approval of the first antisense drug (Fomivirisen, ISIS) for treating a viral infection of the eye marks a milestone in the long-awaited transition of this intriguing technology from the research laboratory to the clinic. However, there are outstanding challenges in antisense therapeutics. Our current work addresses the potential to substantially reduce the dose and toxicity of antisense drugs, and demonstrates successful application of antisense technology to a cellular, rather than viral or oncogenic target. Moreover, they raise the possibility that exploiting injury-associated breaches of the blood-brain barrier [15, 38] to deliver antisense drugs into the brain could place the trauma model in a unique position

to advance the goal of clinical antisense therapeutics for the nervous system.

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References

1. McIntosh TK, Juhler M, Wieloch T (1998) Novel pharmacologic strategies in the treatment of experimental traumatic brain injury. J Neurotrauma 15:731–769
2. Siesjo BK (1993) Basic mechanisms of traumatic brain damage. Ann Emerg Med 22:959–969
3. Gennarelli TA, Graham DI (1998) Neuropathology of the head injuries. Semin Clin Neuropsychiatry 3:160–175
4. Yakovlev AG, Faden AI (1995) Molecular biology of CNS injury. J Neurotrauma 12:767–777
5. Roe SY, McGowan EM, Rothwell NJ (1998) Evidence for the involvement of corticotrophin-releasing hormone in the pathogenesis of traumatic brain injury. Eur J Neurosci 10: 553–559
6. Shohami E, Gallily R, Mechoulam R, Bass R, Ben-Hur T (1997) Cytokine production in the brain following closed head injury: dexanabinol (HU-211) is a novel TNF-alpha inhibitor and an effective neuroprotectant. J Neuroimmunol 72:169–177
7. Kaufer D, Friedman A, Seidman S, Soreq H (1998) Acute stress facilitates long-lasting changes in cholinergic gene expression. Nature 393:373–377
8. Kaufer D, Friedman A, Soreq H (1999) The vicious circle: long-lasting transcriptional modulation of cholinergic neurotransmission following stress and anticholinesterase exposure. Neuroscientist 5:173–183
9. Gorman LK, Fu k, Hovda DA, Becker DP, Katayama Y (1989) Analysis of acetylcholine release following concussive brain injury in the rat. J Neurotrauma 6:203
10. Sapolsky RM (1996) Why stress is bad for your brain. Science 273:749–750
11. Beeri R, Le Novere N, Mervis R, Huberman T, Grauer E, Changeux JP, Soreq H (1997) Enhanced hemicholinium binding and attenuated dendrite branching in cognitively impaired acetylcholinesterase-transgenic mice. J Neurochem 69:2441–2451
12. Crooke ST (1998) An overview of progress in antisense therapeutics. Antisense Nucleic Acid Drug Dev 8:115–122
13. McCarthy MM (1998) Use of antisense oligonucleotides in the central nervous system: why such success? In: Stein CAK, Krieg AM (eds) Applied antisense oligonucleotide technology. Willey-Liss, New York, pp 283–296
14. Seidman S, Eckstein F, Grifman M, Soreq H (1999) Antisense technologies have a future fighting neurodegenerative diseases. Antisense Nucleic Acid Drug Dev 9:333–340
15. Chen Y, Constantini S, Trembovler V, Weinstock M, Shohami E (1996) An experimental model of closed head injury in mice: pathophysiology, histopathology, and cognitive deficits. J Neurotrauma 13:557–568
16. Grisaru D, Lev-Lehman E, Shapira M, Chaikin E, Lessing JB, Eldor A, Eckstein F, Soreq H (1999) Human osteogenesis involves differentiation-dependent increases in the morphogenically active 3' alternative splicing variant of acetylcholinesterase. Mol Cell Biol 19:788–795
17. Casceres A, Steward O (1983) Dendritic reorganization in the denervated gyrus of the rat following entorhinal cortical lesions: a Golgi and electron microscopic analysis. J Comp Neurol 214:387–403
18. McKinney RA, Debanne D, Gahwiler BH, Thompson SM (1997) Lesion-induced axonal sprouting and hyperexcitability in the hippocampus in vitro: implications for the genesis of posttraumatic epilepsy. Nat Med 3:990–996
19. Sternfeld M, Ming G, Song H, Sela K, Timberg R, Poo M, Soreq H (1998) Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. J Neurosci 18:1240–1249
20. Baldwin SA, Gibson T, Callihan CT, Sullivan PG, Palmer E, Scheff SW (1997) Neuronal cell loss in the CA3 subfield of the hippocampus following cortical contusion utilizing the optical disector method for cell counting. J Neurotrauma 14: 385–398
21. Tang YP, Noda Y, Hasegawa T, Nabeshima T (1997) A concussive-like brain injury model in mice. II. Selective neuronal loss in the cortex and hippocampus. J Neurotrauma 14:863–873
22. Andres C, Beeri R, Friedman A, Lev-Lehman E, Henis S, Timberg R, Shani M, Soreq H (1997) Acetylcholinesterase-transgenic mice display embryonic modulations in spinal cord choline acetyltransferase and neurexin Ibeta gene expression followed by late-onset neuromotor deterioration. Proc Natl Acad Sci U S A 94:8173–8178
23. Beeri R, Andres C, Lev Lehman E, Timberg R, Huberman T, Shani M, Soreq H (1995) Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. Curr Biol 5:1063–1071
24. Perry E, Martin-Ruiz C, Lee M, Griffiths M, Johnson M, Piggott M, Haroutunian V, Davis K, Gotti C, Tzartos S, Cohen O, Soreq H, Jaros E, Perry R, Court J (2000) Nictotinic receptor subtypes in human brain ageing and dementia. Eur J Pharmacol (in press)
25. Kaufer D, Friedman A, Seidman S, Soreq H (1998) Acute stress facilitates long-lasting changes in cholinergic gene expression. Nature 393:373–377
26. Grisaru D, Sternfeld M, Eldor A, Glick D, Soreq H (1999) Structural roles of acetylcholinesterase variants in biology and pathology. Eur J Biochem 264:672–686
27. Inestrosa NC, Alvarez A, Perez CA, Moreno RD, Vicente M, Linker C, Casanueva OI, Soto C, Garrido J (1996) Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. Neuron 16:881–891
28. Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 400:173–177
29. Morrow BA, Elsworth JD, Inglis FM, Roth RH (1999) An antisense oligonucleotide reverses the footshock-induced expression of fos in the rat medial prefrontal cortex and the subsequent expression of conditioned fear-induced immobility. J Neurosci 19:5666–5673
30. Grifman M, Soreq H (1997) Differentiation intensifies the susceptibility of pheochromocytoma cells to antisense oligodeoxynucleotide-dependent suppression of acetylcholinesterase activity. Antisense Nucleic Acid Drug Dev 7:351–359
31. Soreq H, Seidman S (2000) Antisense approach to isoform specific blockade of acetylcholinesterase. In: Crooke ST (ed) Antisense drug technology: principles, strategies, and applications. Dekker, New York
32. Xie J, McCobb DP (1998) Control of alternative splicing of potassium channels by stress hormones. Science 280:443–446
33. Kreutz MR, Bockers TM, Bockmann J, Seidenbecher CI, Kracht B, Vorwerk CK, Weise J, Sabel BA (1998) Axonal injury alters alternative splicing of the retinal NR1 receptor: the

preferential expression of the NR1b isoforms is crucial for retinal ganglion cell survival. J Neurosci 18:8278–8291
34. Imai Y, Matsuo N, Ogawa S, Tohyama M, Takagi T (1998) Cloning of a gene, YT521, for a novel RNA splicing-related protein induced by hypoxia/reoxygenation. Brain Res Mol Brain Res 53:33–40
35. Gattoni R, Mahe D, Mahl P, Fischer N, Mattei MG, Stevenin J, Fuchs JP (1996) The human hnRNP-M proteins: structure and relation with early heat shock-induced splicing arrest and chromosome mapping. Nucleic Acids Res 24:2535–2542
36. Tongiorgi E, Righi M, Cattaneo A (1997) Activity-dependent dendritic targeting of BDNF and TrkB mRNAs in hippocampal neurons. J Neurosci 17:9492–9505
37. Robinson RG (1997) Neuropsychiatric consequences of stroke. Annu Rev Med 48:217–229
38. Friedman A, Kaufer D, Shemer J, Hendler I, Soreq H, Tur-Kaspa I (1996) Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nat Med 2:1382–1385

Ref 14

EXHIBIT E

Molecular Psychiatry (2002) 7, 874–885

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ORIGINAL RESEARCH ARTICLE

Neuronal overexpression of 'readthrough' acetylcholinesterase is associated with antisense-suppressible behavioral impairments

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Molecular origin(s) of the diverse behavioral responses to anticholinesterases were explored in behaviorally impaired transgenic (Tg) FVB/N mice expressing synaptic human acetylcholinesterase (hAChE-S). Untreated hAChE-S Tg, unlike naïve FVB/N mice, presented variably intense neuronal overexpression of the alternatively spliced, stress-induced mouse 'readthrough' mAChE-R mRNA. Both strains displayed similar diurnal patterns of locomotor activity that were impaired 3 days after a day-to-night switch. However, hAChE-S Tg, but not FVB/N mice responded to the circadian switch with irregular, diverse bursts of increased locomotor activity. In social recognition tests, controls displayed short-term recognition, reflected by decreased exploration of a familiar, compared to a novel juvenile conspecific as well as inverse correlation between social recognition and cortical and hippocampal AChE specific activities. In contrast, transgenics presented poor recognition, retrievable by tetrahydroaminoacridine (tacrine, 1.5 mg kg^{-1}). Tacrine's effect was short-lived (<40 min), suggesting its effect was overcome by anticholinesterase-induced overproduction of mAChE-R. Consistent with this hypothesis, antisense oligonucleotides (two daily intracerebroventricular injections of 25 ng) arrested mAChE-R synthesis, selectively reduced mAChE-R levels and afforded an extended (>24 h) suppression of the abnormal social recognition pattern in transgenics. Efficacy of antisense treatment was directly correlated with AChE-R levels and the severity of the impaired phenotype, being most apparent in transgenics presenting highly abnormal pretreatment behavior. These findings demonstrate that neuronal AChE-R overproduction is involved in various behavioral impairments and anticholinesterase responses, and point to the antisense strategy as a potential approach for re-establishing cholinergic balance.
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Keywords: antisense; circadian rhythm; mouse; psychological stress; social recognition; transgenic

Introduction

Social behavior is a complex phenotype, composed of the individual's general level of activity, cognitive perception and anticipation of the outcome of such behavior.[1] Working and storage memory and the ability to integrate information can also contribute towards social behavior, which is tightly linked to cholinergic neurotransmission. For example, the hypocholinergic features of Alzheimer's disease (AD) patients include aggressive behavior and/or avoidance of novel social challenges,[2] as well as fears of social interactions alleviated by treatment with anticholinesterases.[3] Surprisingly, anticholinesterases, eg tacrine (tetrahydroaminoacridine, Cognex, Parke-Davis), donepezil (Aricept, Pfizer), rivastigmine (Exelon, Novartis) and galantamine (Reminyl, Janssen), were reported to cause more pronounced improvement in more severely affected patients. To explain the molecular basis of this phenomenon, re-evaluation is needed of the linkage between cholinergic neural pathways, social behavior and acetylcholinesterase (AChE).

Both anticholinesterase exposure and stressful insults, ie confined swim, induce in the mammalian brain a rapid c-fos elevation that mediates muscarinic responses and subsequent *ACHE* overexpression.[4] A stress-associated switch in alternative splicing[5,6] diverts AChE from the major, 'synaptic' AChE-S to the normally rare 'readthrough' AChE-R variant.[7] The distinctive non-catalytic activities of these AChE isoforms,[8] suggest links between AChE-R accumulation and behavioral anticholinesterase responses.

Transgenic (Tg) mice overexpressing human (h) AChE-S in brain neurons are amenable to pursuit of this linkage. These mice present early-onset loss of learning and memory capacities,[9] progressive dendritic depletion,[10] stress-related neuropathology,[8] and modified anxiety responses.[11] However, their social

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behavior and psychological stress responses have not yet been addressed.

AChE-S Tg mice constitutively overexpress AChE-R mRNA in their intestinal epithelium. When exposed to an organophosphate anticholinesterase (DFP), they fail to increase further the already overproduced AChE-R, and present extreme DFP sensitivity. Humans with inherited AChE overexpression are likewise hypersensitive to the anticholinesterase pyridostigmine.[12] Our working hypothesis postulated that at appropriate levels, AChE-R accumulation in response to stress restores normal cholinergic activity and social behavior. However, under chronic stress, acute anti-AChE treatment or exposure, or in individuals with inherited AChE excess, AChE-R increases to a limit beyond which their cholinergic system cannot further respond and impaired social behavior is a result.

To test this hypothesis, we ascertained whether: (a) AChE-S Tg mice display excessive response to a mildly stressful stimulus, a switch in the day/night cycle;[13] (b) examined AChE-R expression in the brain neurons of AChE-S Tg mice; and (c) studied the social recognition behavior[14] of AChE-S Tg mice before and after administration of tacrine or AS3, an antisense oligonucleotide (AS-ON) shown to selectively suppress AChE-R production.[15] Our findings demonstrate constitutive mAChE-R accumulation with inter-animal variability in brain neurons of hAChE-S Tg mice, associated with an exaggerated response to changes in circadian rhythm, and impaired social recognition, which are amenable to effective AS-ON suppression.

Materials and methods

Animals

AChE-S Tg mice were obtained in a 100% FVB/N genotype from heterozygous breeding pairs.[9] Control, non-Tg FVB/N mice were obtained by littermate breeding. Adult, 8–20 wk old Tg and control male mice were housed 4–5 per cage in a 12 h dark/light cycle with free access to food and water. All experiments were conducted during the first half of the dark phase of a reversed 12 h dark/light cycle, under dim illumination. Routine locomotor activity in the home cage was measured using a remote motility detector (MFU 2100, Rhoma-Labortechnik, Hofheim, Germany) to quantify changes in the electromagnetic field.

Telemetric measurements

Battery operated biotelemetric transmitters (model VM-FH, Mini Mitter, Sun River, OR, USA) were implanted in the peritoneal cavity under ether anesthesia 12 days prior to the test. After implantation, mice were housed in separate cages with free access to food and water. Output was monitored by a receiver board (model RA-1010, Mini Mitter) placed under each animal's cage and fed into a peripheral processor (BCM 100) connected to a desktop computer. Locomotor activity after the dark/light shift was measured by detecting changes in signal strength as animals moved about in their cages, so that the number of pulses that were generated by the transmitter was proportional to the distance the animal moved. The cumulative number of pulses generated over the noted periods was recorded.[16] Recording lasted 24 consecutive h, starting at 9:30 am, with the light phase of a 12:12 h dark/light cycle beginning at 7:00 a.m. To initiate a day/night switch, the dark/light periods were reversed and recording started 72 h after the switch and lasted 24 h. Following intraperitoneal injection of AS-ONs (see below), recording proceeded for an additional 3 h.

Social exploration tests

Each mouse was placed in a semicircular, transparent observation box and allowed 15 min for habituation, following which a juvenile male mouse (23–29 days old) was introduced. The time spent by the experimental mouse in social exploration consisted mainly of body and anogenital sniffing, chasing, attacking and crawling over the juvenile. Measurements covered a 4-min period, using a computerized event recorder. Each mouse underwent two successive social exploration sessions at the noted inter-session intervals. The first session was considered as baseline. In the second session (test), either the same or a different juvenile was introduced. Social recognition was calculated as a percentage of tested out of baseline exploration time recorded for each mouse.

In situ hybridization and AChE activity measurements

Animals were killed by cervical dislocation, and brains were removed and dissected or fixed for *in situ* hybridization. AChE activity was measured in hippocampus, cortex and cerebellum extracts as described.[17] Protein determination was performed using a detergent-compatible kit (DC, Bio-Rad, München, Germany). Immunoblot detection of specific AChE isoforms was as reported.[16]

For *in situ* hybridization, 5 μm paraffin sections of brain tissue were prepared after fixation by transcardial perfusion of anaesthetized mice with 4% paraformaldehyde in PBS (pH 7.4). A 50-mer fully 2′-O-methylated 5′-biotinylated AChE-R cRNA probe was applied as described.[4] Following probe detection with a streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia Biotech, Little Chalfont, UK) and Fast Red as the reaction substrate (Roche Diagnostics, Mannheim, Germany), micrographs of hippocampal and cortical neurons were subjected to semi-quantitative evaluation of Fast Red staining. Mean signal intensities of light micrographs (taken with a Real-14 color digital camera, CRI, Boston, MA, USA) were analyzed using Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA) image analysis software. The mean intensity of Fast Red labeling was measured in CA3 hippocampal and cortical neurons and corrected for background staining in each picture.

Immunocytochemistry

Immunocytochemistry of glial fibrillary acidic protein (GFAP) was performed as described.[6] Briefly, floating,

formalin-fixed, 30 μm, coronal cryostat-sections were pretreated with trypsin (type II, Sigma Chemical Co, St Louis, MO, USA) 0.001% for 1 min. Sections were incubated overnight at 4°C with a mouse anti-glial fibrillary acidic protein (GFAP) antibody (clone GA-5, Sigma-Israel, Rehovot, Israel), diluted 1:500. Then sections were incubated overnight at 4°C with horseradish peroxidase-labeled goat-anti-mouse antibody (Sigma-Israel), diluted 1:100. Color was developed by reaction with diaminobenzidine 0.0125%, nickel ammonium sulfate 0.05% and hydrogen peroxide (0.00125%). The development time of the DAB reaction product was controlled by stopwatch to ensure comparability between experiments. Sections were counterstained with cresyl violet.

Quantitative analysis of the hippocampal stratum lacunosum moleculare (SLM) was performed at the level of posterior 2.5 mm from bregma. Using a 40× objective, consecutive fields of the SLM were visualized with a Nikon microscope and processed using an AnalySIS image analysis system. A total of 35 astrocytes were sampled from each group (control *vs* Tg). The variables that were compared were intensity of staining of the soma (arbitrary units from a range of 256 shades of gray), soma size (μm^2), and thickness of the largest process of each astrocyte at the process stem (μm). Statistical comparisons were made using Student's *t*-test, with 68 degrees of freedom.

Considerations for designing antisense tests

In vivo antisense suppression of *de novo* AChE-R synthesis was employed throughout the current study to provide a proof of concept (ie demonstrate the causal involvement of the secretory, soluble mAChE-R variant in the excessive locomotor activity and the impaired social recognition of Tg mice). Two types of experiments were performed: (1) intracerebroventricular (i.c.v.) AS3 injection of animals subjected to longitudinal social exploration tests (up to 1 week post-treatment); and (2) i.c.v. injection followed by 1-day social exploration test, immunochemical detection and measurement of catalytic activity of brain AChE. Both of these were associated with certain inherent limitations, as detailed below, yet each test provided evidence to support part of the explored concept.

Intracranial AS-ON injection is inherently more powerful when centrally controlled behavioral parameters are sought; limitations in this case involve the duration of tests (as the animals are all at a post-surgery state) and the requirement to control for the outcome of this surgical procedure in addition to the behavioral test itself. To avoid excessive complications, we refrained from employing double operations (and, therefore, could not use telemetric measurements, which require transmitter implantation, on i.c.v.-injected animals).

The experimental controls, as well, were chosen after careful consideration. Each test should involve both Tg and control animals, as well as sham treatment (injection of either saline or an irrelevant oligonucleotide) and comparison between pre- and post-treatment phenotypes. Whenever possible, animals were self-compared, requiring careful time-of-day comparisons; in other cases, groups of animals with similar pre-treatment behavior patterns were compared to each other with regard to the efficacy of the antisense treatment. Neither of these tests is conclusive by itself, however, their cumulative outcome substantially supported the possibility of employing antisense knockdown in careful behavioral tests.

Cannula implantation

Mice under sodium pentobarbital anesthesia (50 mg kg^{-1}, i.p.) were placed in a stereotaxic apparatus. Skulls were exposed and a burr hole was drilled. Implantation was with a 26-gauge stainless steel guide cannula (Plastics-One, Roanoke, VA, USA). The tip of the guide cannula was positioned 1 mm above the left lateral ventricle according to the following coordinates: A: −0.4 −0.66 (bl-3.8), (bl = bregma–lamda); L: 1.5; D: −2.2. The guide cannula was secured to the skull with three stainless-steel screws and dental cement, and was closed by a dummy cannula. Mice were housed in individual cages and allowed postoperative recovery of 10–14 days before experiments.

Preparation of AS-ON

For i.c.v. injection, 2′-O-methyl protected (three-3′ nucleotides) oligonucleotides (5 μM) targeted against murine AChE (AS3) or BuChE (ASB) mRNA[15] were combined with 13 μM of the lipophilic transfection reagent DOTAP (Roche Diagnostics) in PBS and incubated for 15 min at 37°C prior to injection. One μl (25 ng) of this oligonucleotide solution was injected in each treatment.

I.c.v. administration of AS-ON

For intracranial microinjections, solutions were administered through a 33-gauge stainless steel internal cannula (Plastic One), which was 1 mm longer than the guide cannula. A PE20 tube connected the internal cannula to a microsyringe pump (KD Scientific Instruments, Boulder, CO, USA). Solutions were administered at a constant rate for 1 min, followed by 1 min during which the internal cannula was left within the guide cannula, to avoid spillage from the guide cannula. Correct positioning of the cannula was verified following each experiment by injection of trypan blue through the cannula and testing dye distribution after removal of the brains.

Statistical analysis

The results of the *in situ* hybridization experiment were analyzed by a *t*-test. The results of the circadian shift were analyzed by a three-way, repeated measures ANOVA (genotype × day (routine/reversed) × circadian phase (dark/light)). The results of the social recognition test were analyzed by a three-way, repeated measures ANOVA (genotype × stimulus animal (same or different juvenile) × intersession interval). The results of the experiment on tacrine's effect on social recognition were analyzed by a three-way ANOVA (genotype ×

stimulus animal (same or different juvenile) × drug (tacrine/saline)). The results of the effect of AS3 on social recognition were analyzed by a two-way, repeated measures ANOVA (pretreatment (short/long explorers) × time (days after injection)). The results of the specificity of AS3 effect were analyzed by a three-way ANOVA (genotype × drug (AS3/ASB) × time (before/after the treatment)). All ANOVAs were followed by post-hoc tests with the Fisher PLSD procedure.

Results

Transgenic mice overexpress host AChE-R

To explore the specific contribution of variant AChE mRNA transcripts towards neuronal *ACHE* gene expression, Tg mice overexpressing hAChE-S in the nervous system were tested by high resolution *in situ* hybridization using cRNA probes selective for each of the two major AChE variants. Excessive labeling was observed in hAChE-S Tg mice as compared with controls in which hybridization was performed with the AChE-S selective probe. This was consistent with the expected cumulative contribution of the overexpressed human transgene and the host mouse (m)AChE-S mRNA transcript.[9,10] However, hAChE-S Tg mice also displayed variably excessive labeling with the AChE-R cRNA probe, decorating mouse (m)AChE-R mRNA. Figure 1 presents a representative micrograph of mAChE-R mRNA overexpression in the cortex and hippocampus of a Tg as compared to a control mouse.

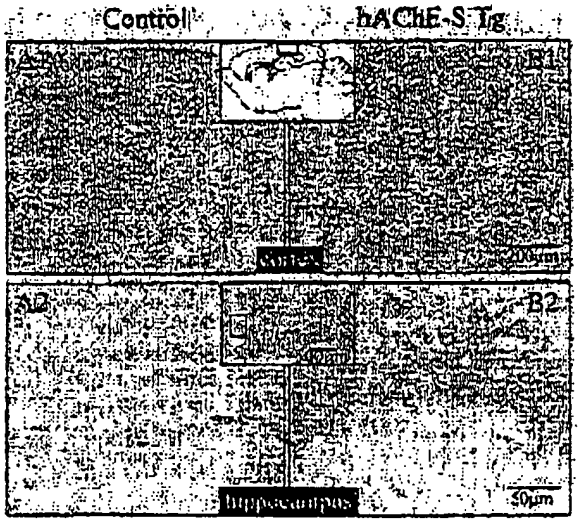

Figure 1 Intensive neuronal AChE-R mRNA expression in Tg mice. Shown are representative micrographs of *in situ* hybridization in parietal cortex (A_1, B_1) and hippocampus (A_2, B_2) using an AChE-R cRNA probe and Fast Red detection. Insets: schematic drawing (top) or low-magnification micrograph (bottom) presenting the location of higher magnification micrographs in the cortex and hippocampal CA3 region. Note the intense AChE-R expression in cortical and hippocampal neurons of Tg mice.

Neuronal mAChE-R mRNA overproduction in these Tg mice was heterogeneous in its extent, yet significantly higher than that in control mice. Analysis of Fast Red staining showed an increase from an average of 20 ± 3 arbitrary intensity units (± standard error of the mean, SEM) in five control animals to 41 ± 5.5 in six transgenics ($t(9) = 10.27$, $P < 0.05$). This suggested an inherited predisposition to constitutive AChE-R overproduction in Tg mice. Because AChE-R overproduction is associated with psychological stress, this further called for evaluating its neuroanatomical and behavioral manifestations.

Hypertrophy in hippocampal astrocytes reflects elevated stress in hAChE-S Tg mice

Immunocytochemical labeling of glial fibrillary acidic protein (GFAP) was used in search of an independent parameter for evaluating the stress-prone state of hAChE-S Tg mice. Hippocampal astrocytes are known from previous studies to be sensitive to various forms of stress.[16,18] This property is manifest in morphological changes, increased size of cell soma and of astrocytic processes collectively called 'hypertrophy' and which is accompanied by increased expression of GFAP. The hippocampal SLM is particularly enriched in astrocytes, which appeared to be hypertrophic in hAChE-S Tg mice (Figure 2b, d) as compared to age-matched controls (Figure 2a, c). Astrocytes in other regions, such as cortex appeared unchanged (Figure 2e, f). Figure 2 presents this selective hippocampal change, which is generally considered to reflect the cumulative load of stressful insults in the mammalian brain and

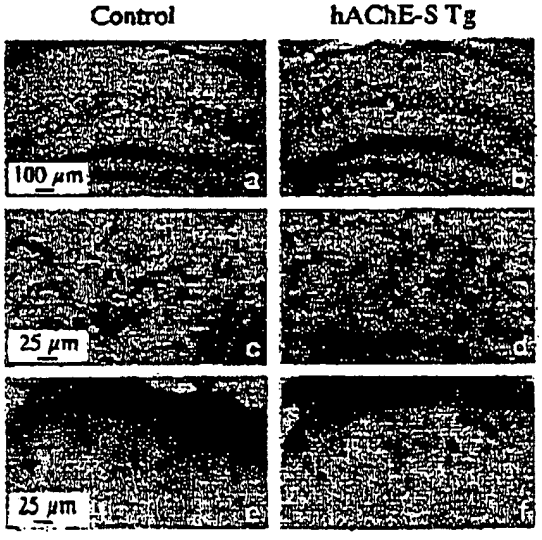

Figure 2 Intensified GFAP staining in hypertrophic hippocampal astrocytes of hAChE-S Tg mice. Shown are light microscopy micrographs of GFAP immunocytochemical staining in the brain of control (a, c, e) and Tg (b, d, f) mice. Note the intensified cell body staining in hippocampal (a–d) but not in cortical astrocytes (e, f) and the thickened process extensions in the transgenics' astrocytes.

is frequently associated with impaired cognitive and behavioral properties.[20]

Intensity of staining of GFAP-like immunoreactivity in the astrocytic soma was significantly higher in Tg mice (176.3 ± 4.0, arbitrary units) compared to control mice (147.8 ± 3.5, $t = 5.3$, $P < 0.0001$). A cross-sectional area of the astrocytic soma was significantly increased in Tg (45.9 ± 1.4 μm^2) compared to control mice (35.4 ± 1.5, $t = 5.16$, $P < 0.0001$). The stem thickness of the large astrocytic process was greater in Tg mice (1.75 ± 0.05 μm) compared to control mice (1.32 ± 0.05, $t = 6.21$, $P < 0.0001$). Taken together, these data form a picture of 20–30% hypertrophy in hippocampal astrocytes of hAChE-S Tg mice, consistent with the earlier reports of stress-associated and pathology-associated astrocytic hypertrophy.[16,18]

AChE overexpression predisposes to hypersensitivity to changed circadian cycle

Behavioral differences between Tg and control mice were first sought by recording locomotion patterns. Under routine conditions, both genotypes displayed similar home cage activity (Figure 3a). Their circadian rhythms included, as expected, significantly more frequent and pronounced locomotor activity during the dark phase of the circadian cycle ($F(1,24) = 18.16$, $P < 0.001$) (summarized in Figure 3c). Seventy-two hours following reversal of the light/dark phases, both genotypes lost most of the circadian rhythm in their locomotor activity, as reported by others,[21] but presented distinctive behavioral patterns (Figure 3b and c). After the shift, hAChE-S Tg mice showed a general increase in activity, which was reflected in a significant genotype by day (routine *vs* reversed) interaction ($F(1,24 = 4.68$, $P < 0.05$). Post-hoc tests demonstrated in Tg mice significantly increased activity in the reversed cycle (compared with activity in the routine cycle), both during the dark and the light phases ($P < 0.05$). In addition, activity in the dark phase of the reversed cycle, was significantly greater in Tg compared with control mice. These findings indicate that adjustment to the circadian insult was markedly impaired in Tg mice, suggesting that these mice display a genetic predisposition to abnormal responses to changes in the circadian rhythm. The transgenics' intensified activity was found to be suppressed for a short time (<3 h) by i.p. administration of AS-ONs targeted to the common domain shared by all AChE mRNA variants (preliminary data, data not shown).

Impaired social recognition due to AChE excess

In the social recognition paradigm, control mice could recognize a previously encountered ('same') juvenile. This is manifest as a reduction in exploration time in the second exposure of the mice to the same, but not to a different juvenile, provided that the time interval from the end of the first encounter with that juvenile to the beginning of the memory test did not exceed 15 min. As expected, this memory decayed with increased intersession interval. In contrast, Tg mice tended to explore the previously introduced juvenile longer than control mice and did not display social recognition even after a short interval of 5 min (Figure 4). These findings were reflected by a significant statistical interaction between the genotype (control *vs* Tg) and the stimulus juvenile (same/different) ($F(1,76) = 9.93$, $P < 0.01$). In Tg mice, post-hoc analysis revealed significant reduction in exploration time only when Tg mice were tested immediately after the baseline (0 interval) with the same juvenile. These results are consistent with the cholinergic modulation of social recognition behavior.[22]

The reversible AChE inhibitor, tacrine, has been clinically used for blocking acetylcholine hydrolysis and extending the impaired memory of Alzheimer's disease patients.[3] Therefore, we tested the capacity of tacrine (1.5 mg kg^{-1}), injected immediately following a baseline encounter with a juvenile mouse, to improve the social recognition of Tg mice. Injected mice were tested with either the same or a different juvenile following a 10-min interval. As expected from previous reports on the beneficial effects of tacrine on social recognition in rats,[23] injection of Tg mice with tacrine induced a significant improvement in recognition memory, with post-treatment performance similar to that displayed by untreated control mice. In contrast, Tg mice displayed no recognition of the same juvenile when injected with saline, and non-Tg control mice maintained unchanged recognition performance when injected with either tacrine or saline (Figure 5). These findings were reflected by a significant three-way interaction between the genotype (control/Tg), the stimulus juvenile (same/different) and the drug (tacrine/saline) ($F(1,52) = 4.18$, $P < 0.05$). In a similar experiment, in which the injections preceded the social recognition test by 40, rather than 10 min, tacrine had no effect on either Tg or control mice (data not shown). Therefore, tacrine facilitated memory consolidation when administered during the consolidation process, but did not affect acquisition of memory when given in advance.

Explorative behavior is inversely correlated with brain AChE activity

Apart from its improvement of memory, tacrine suppressed the exploration behavior toward a different juvenile in control ($P < 0.05$) but not in Tg mice. Decreased locomotor activity under tacrine treatment was suggested to reflect cholinergic mediation of social exploration behavior.[24] To further investigate this concept, control mice were divided into three equal groups ($n = 10$), presenting short, intermediate or long exploration time of the same juveniles. AChE activity was determined in the cortex and hippocampus of each subgroup, 24 h following social recognition tests of the 'same' juvenile (presented 10 min following first exposure). Mice with lower levels of cortical and hippocampal AChE activity spent more interaction time with the 'same' juvenile than mice with high AChE activity levels (Table 1), so that their explorative behavior was inversely correlated with cortical and hippocampal AChE activity levels (correlation magnitude, $r = -0.49$ and 0.41, respectively). Compared to

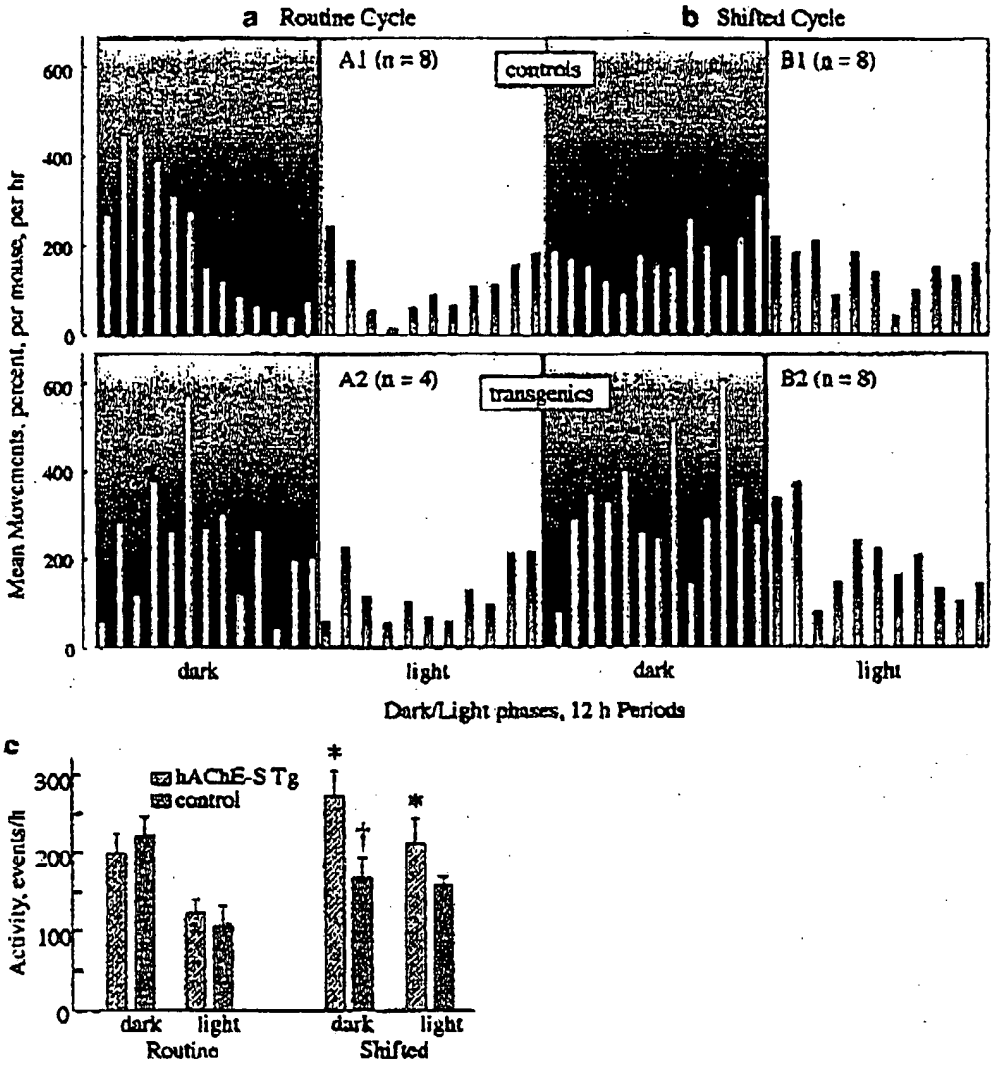

Figure 3 Spontaneous locomotor activity of control and Tg mice under routine dark/light cycle and following cycle reversal. Locomotor activity was detected by biotelemetric-recorded movements for 24 h and is displayed as percent of the mean movements per mouse per h. Shaded areas in the figure indicate the dark phases of the light/dark cycle. (a) Routine cycle; (b) shifted cycle; (c) summated activity intensification. Shown are values of locomotor activity during the dark and light phases in the daily cycle for Tg and age-matched control mice under routine and reversed cycles. *Significantly different from the corresponding group in the routine condition ($P < 0.05$). †Significantly different from Tg mice in the dark phase of the reversed condition.

shorter explorers, longer explorer mice exhibited a 29% reduction in cortical AChE activity, corresponding to a >180% increase in social exploration time. The significance of the difference between the shorter and longer explorers was verified by ANOVA ($F(2,27) = 4.89$, $P < 0.05$) and post-hoc tests.

The lack of tacrine effect on the social recognition performance in control mice, and its improvement effect on the social recognition in transgenics, with approximately 50% excess AChE,[8] presented an apparent contradiction to the inverse correlation between AChE catalytic activity and social exploration. One potential explanation to this complex situation was that the inverse correlation in control mice reflected primarily the levels of the synaptic enzyme AChE-S; in contrast, the massive mAChE-R excess in the Tg brain could cause their impaired social recognition behavior. According to this working hypothesis, selective suppression of AChE-R should improve the social recognition performance. To test this hypothesis, we adopted i.c.v. injection of AS3 to prevent *de novo* mAChE-R production.[16] Mice were tested in the social exploration paradigm once before (baseline) and then 1, 3 and 6 days after two daily injections of AS3. Figure 6a presents the experimental design of these tests.

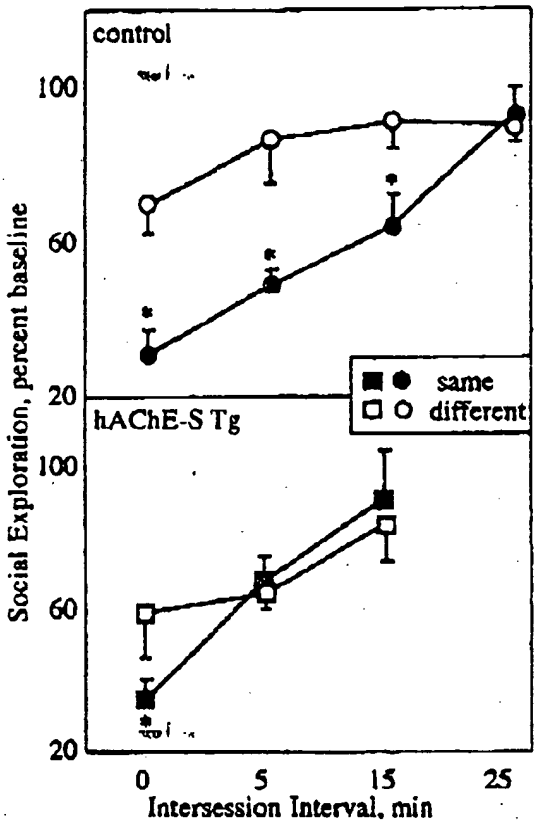

Figure 4 Working memory deficiency in Tg mice. Shown is the percent of baseline social exploration time for 8–11 wks old Tg and control male mice as a function of the intersession interval. Average baseline exploration time was 143 ± 5 s and 153 ± 5 s (± SEM) for transgenics (n = 42) and control mice (n = 48), respectively. Asterisks mark significant reductions of exploration time toward the same juvenile ($P < 0.05$), as compared to a different juvenile, ie short-term working memory. Increased exploration time of the same juvenile with increasing intersession intervals reflects time-dependent decay in the working memory of control mice. After a 5-min interval, Tg mice displayed no reduction in exploration time toward the same juvenile, indicating that they did not remember the same mouse for even 5 min.

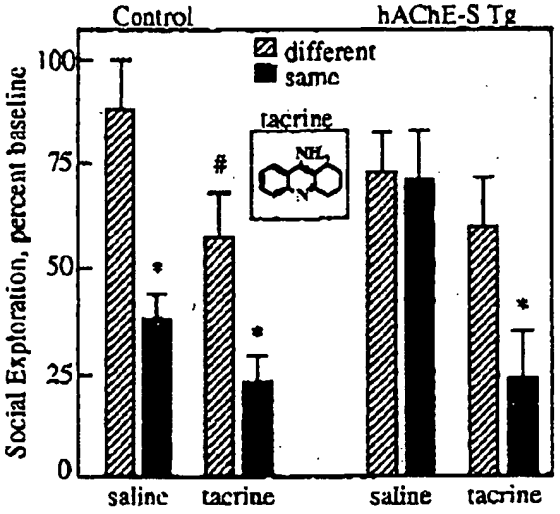

Figure 5 Tacrine improves working memory of Tg mice. Presented is the mean social exploration time ± SEM as a percentage of baseline time for 29 control and 32 Tg mice (12–15 wks old, 7–8 mice per group) where either tacrine (1.5 mg kg^{-1}) or saline (10 ml kg^{-1} body weight) was administered intraperitoneally immediately following the baseline exploration period. The intersession interval was 10 min for all groups. Note the post-treatment shortening of explorative time of Tg mice, reflecting improved working memory. Asterisks mark significant reduction of exploration time toward the same juvenile ($P < 0.05$) as compared to a different juvenile. # Marks a significant tacrine-induced reduction of exploration time toward a different juvenile ($P < 0.05$).

AS3 improvement of social exploration increases in efficacy and duration in animals with severe pre-treatment impairments

Post-treatment follow-up of social exploration was performed 1, 3 and 6 days following AS3 treatment in animals with short, medium and long pre-treatment social exploration behavior (n = 5–6 per group). As expected, there was a significant overall difference between the short and the long groups in exploration time ($F(1,24) = 10.81$, $P < 0.05$). However, post-hoc tests revealed that these groups differed significantly only during the pre-treatment day ($P < 0.05$), and not after the AS3 treatment (Figure 6b). Furthermore, within the long, but not the short explorers group, social exploration of the 'same' juvenile was significantly reduced ($P < 0.05$) 1 day after the AS3 injection, with progressive increases in social exploration time during the 5 subsequent days. Because of the pre-treatment differences, the severely impaired animals sustained a certain level of improvement even at the sixth post-treatment day (Figure 6b) (ie, even on this day there was no resumption of the pre-treatment difference between the short and long explorers). This experiment thus demonstrated both the efficacy and the reversibility of the antisense treatment, however with exceedingly long duration, especially in animals with severe pre-treatment impairments and in comparison to the short-term efficacy of tacrine.

Antisense AChE-R mRNA suppression selectively reduces brain AChE-R protein

Tg mice with long pre-treatment explorative behavior displayed a significant improvement in social exploration of the 'same' juvenile 24 h following the second treatment with AS3, but not with the irrelevant AS-ON ASB ($F(1,10) = 33.95$, $P < 0.001$). ASB, targeted to the related enzyme, butyrylcholinesterase, served as a sequence specificity control. Control mice with either long or short pre-treatment social exploration showed no response to either AS3 or ASB (Figure 7a and data not shown).

Table 1 Social exploration behavior and brain AChE activities[a]

	Exploration time (percent of baseline)	*Specific AChE activity (nmol ATCh hydrolyzed min⁻¹ mg protein⁻¹)*	
		Hippocampus	*Cortex*
Total population	80 ± 4	87 ± 4	90 ± 5
Shorter exploration time	57 ± 3	96 ± 6	104 ± 10
Intermediate exploration time	78 ± 2	95 ± 6	92 ± 10
Longer exploration time	104 ± 3	71 ± 7**	74 ± 8*

[a]Control FVB/N male mouse population ($n = 30$) (3–5 months) was divided into three equal groups ($n = 10$) with short, intermediate and long exploration time of the same juvenile (shown as average ± SEM percent of baseline). Intersession interval was 10 min for all groups. Mice were killed 24 h after the behavior test and AChE specific activities were measured in hippocampus and cortex extracts. Asterisks mark significantly lower AChE specific activity in control long explorers as compared with short explorers ($P < 0.01$ for hippocampus and $P < 0.05$ for cortex; ANOVA followed by post-hoc tests with the Fisher PLSD procedure).

Catalytic activity measurements performed 24 h after the last AS-ON injection failed to show differences, perhaps due to the limited number of animals and the variable enzyme levels. However, immunodetected AChE-R protein levels were significantly lower in AS3 treated mice as compared with ASB treated mice, regardless of their genotype or pre-treatment behavior pattern (Figure 7b and data not shown, $F(1,22) = 19.63$, $P < 0.001$). In contrast, densitometric analysis of immunodetected total AChE protein (detected by an antibody targeted to the N-terminus, common to both isoforms) revealed essentially unchanged signals (data not shown). In further tests for potential association, post-treatment AChE-R levels were plotted as a function of the social exploration values. Data points clustered separately before the AS-ON treatment (Figure 8a), with both AChE-R levels and exploration times of controls clearly different from transgenics. After treatment, long explorer transgenics shifted to short exploration values (Figure 8b). Intriguingly, the explorative behavior of long explorer controls was not affected by the treatment, indicating that the reduction in mAChE-R following AS3 treatment affected only animals that were behaviorally impaired before the treatment. Together, these findings attest to the selectivity of the antisense treatment for treating AChE-R overexpressing animals and its sequence-specificity in reversing the AChE-R induced impairment of behavior.

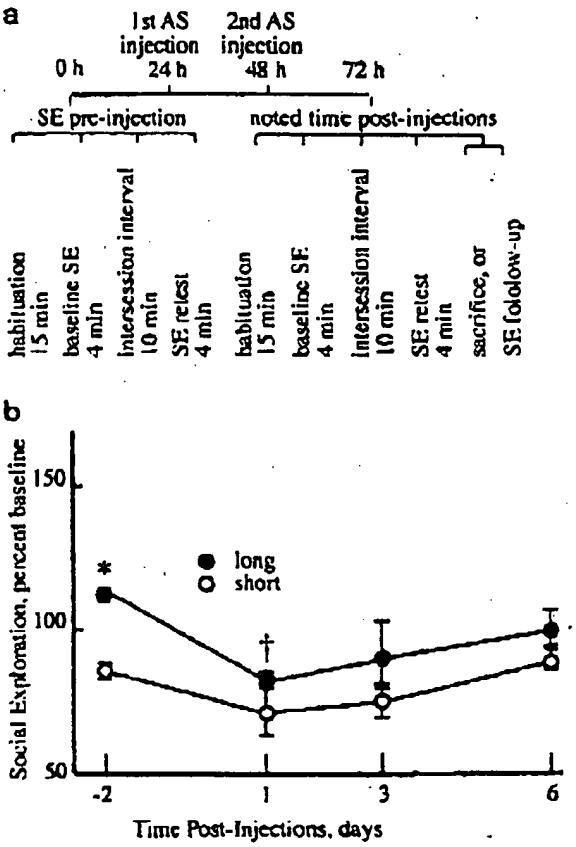

Figure 6 I.c.v. antisense effect on the excessive social exploration behavior of Tg mice. (a) The experimental paradigm. Shown is the order of procedures and tests of the social exploration capacity in cannula-implanted mice following antisense treatment. See Materials and Methods for details. (b) Long-term reversibility and correlation of treatment efficacy with the severity of pre-treatment symptoms. Shown are social exploration values, in percent of baseline performance, for cannulated hAChE-S Tg mice with short and long pre-treatment exploration of the 'same' juvenile, following i.c.v. AS3 treatment ($n = 5$ mice per group). Note that both the efficacy and the duration of the suppression effect are directly correlated with the severity of pre-treatment symptoms. *Significantly different from mice with short exploration time, at day −2 ($P < 0.05$). †Significantly different from mice with long exploration time, at day −2 ($P < 0.05$).

Discussion

Combination of behavioral, molecular and biochemical analyses revealed multileveled contributions of cholinergic neurotransmission and *ACHE* gene expression, towards the general activity and social behavior of adult Tg mice over-expressing neuronal AChE. In addition to inherited excess of hAChE-S, these Tg mice display conspicuous yet heterogeneous overexpression of the stress-associated 'readthrough' mAChE-R in their cortical and hippocampal neurons. Nevertheless, they present close to normal activity patterns under normal maintenance conditions with minimal external chal-

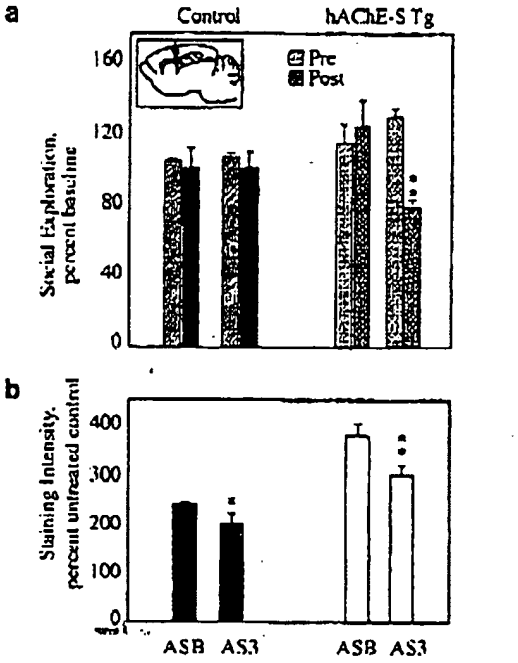

Figure 7 AS3 decreases brain AChE-R levels and ameliorates social recognition deficits in Tg-mice. Tg ($n = 36$) and control ($n = 22$) cannula-implanted mice, 10–20 wks old, were injected i.c.v. with AS-ONs targeted against AChE (AS3) or butyrylcholinesterase (ASB) on 2 consecutive days. Social exploration of the 'same' juvenile was tested 24 h before (pre) and 24 h after (post) injections. Mice were killed immediately after the last social recognition test and brain homogenates subjected to immunodetection of AChE-R. (a) Social exploration behavior. Shown are mean social exploration of the same juvenile (percent of baseline ± SEM) before (pre) and 24 h after (post) AS-ON treatment for long explorer mice (see Table 1). Asterisks mark significant reduction of social exploration time after AS3 treatment ($P < 0.05$). Inset: Location of i.c.v. cannula in the brain (arrow). (b) Immunodetected AChE-R. Mean ± SEM densitometry values for immunodetected AChE-R in cortex extracts of the noted groups post-treatment. AChE-R levels in uncannulated control mice were considered 100%. Asterisks mark significant reduction of AChE-R levels in AS3 as compared to ASB treated mice (**$P < 0.05$, *$P < 0.1$). Note the significant reduction of immunodetected AChE-R and fragments thereof in cortices from both groups treated with AS3 as compared with those treated with the control reagent, ASB.

lenges. In contrast, their capacity to adjust to behavioral changes in response to external signals appears to be compromised, suggesting that they suffer genetic predisposition for adverse responses to stressful stimuli.[20]

Behavioral and learning impairments of cholinergic origin

When subjected to a day/night switch, hAChE-S Tg mice respond with excessive bursts of locomotor activity, particularly during the dark phase, but also during the light phase of the post-shift diurnal cycle.

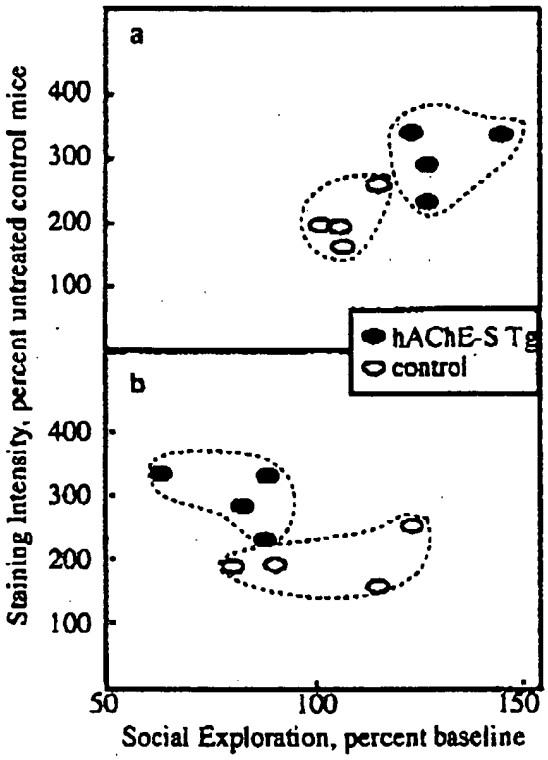

Figure 8 Decreased AChE-R levels correlate with reduced social exploration time in Tg mice. Presented are cortical AChE-R levels (immunodetected protein, percent of levels in uncannulated controls) as a function of social exploration for each mouse before (a) and after (b) AS3 treatment. Long-explorer mice, each represented by a dot, were sorted by their genetic backgrounds (control and Tg). Note the exclusive post-treatment shift in the clustered distribution of the Tg long explorers, with excessive mAChE-R levels, as compared with the non-shifted cluster of long-explorer controls.

In preliminary experiments, this excessive activity could be transiently suppressed by antisense oligonucleotides, which was especially encouraging in view of the progressively impaired neuromotor functioning in these mice.[25] Matched controls, unlike transgenics, display, as expected, relatively suppressed locomotor activity during the post-shift dark phase.[26] When confronted twice with a conspecific young mouse, hAChE-S Tg mice spend significantly longer periods than controls in the social interactions characterizing such confrontations. Similarly, in a new environment, hAChE-S Tg mice displayed increased locomotor activity as compared with controls.[11] In the social recognition paradigm, they failed to remember a conspecific juvenile, even following a delay interval of only 5 min. This extends previous reports on their spatial learning and memory impairments[9,10] and agrees with previous reports[22,27] on the social behavior changes associated with cholinergic impairments.

Several other neurotransmission systems, eg vasopressin,[20] are most likely related, as well, with impaired

social interactions. In hAChE-S Tg mice, however, this phenotype may be attributed to hypocholinergic functioning due to AChE excess, as is evident from the capacity of the AChE inhibitor tacrine to retrieve their social recognition. Nevertheless, tacrine's effects appeared surprisingly short-lived, consistent with findings of others.[29] In contrast, exceedingly low doses of oligonucleotides suppressing AChE-R synthesis exerted considerably longer-term improvement of the social recognition skills of Tg mice. This suggested non-catalytic activities as an alternative explanation(s) for the behavioral and cognitive impairments caused by AChE-R excess.[30]

Circadian switch as a behavioral stressor

Cholinergic neurotransmission circuits are known to be subject to circadian changes[1] and control the sensorimotor cortical regions regulating such activity.[31] Therefore, the intensified response of hAChE-S Tg mice to the circadian switch suggested that their hypocholinergic state is the cause. The variable nature of the excessive locomotor activity in the Tg mice indicates an acquired basis for its extent and duration. A potential origin of such heterogeneity could be the variable extent of neuronal mAChE-R mRNA in the sensorimotor cortex and hippocampal neurons. Both psychological[6] and physical stressors[16] induce neuronal AChE-R overproduction. Exaggerated stress responses, such as the intense locomotor response to the mild stress of a circadian switch, can hence be expected to exacerbate the hypocholinergic state of these already compromised animals.

In social behavior tests, hAChE-S Tg mice display impaired recall processes causing poor recognition when confronted with a conspecific young mouse. Therefore AChE-R overexpression, which is also induced under stress,[6] may be causally involved with the reported suppression of recall processes under stress[32] as well as with the apparent correlation between stress and hippocampal dysfunction.[33] This suggests that excess AChE-R can simultaneously impair recall processes and induce excessive locomotion. Stress-induced effects on learning and memory processes have been reported by others,[34] but were not correlated with AChE levels. Our current findings of improvement in trangenics' exploration behavior following tacrine injection, which would be expected to augment cholinergic neurotransmission, strongly indicate that their hypocholinergic state was the cause.

Advantages and limitations of anticholinesterases

In control mice, with low AChE-R levels, tacrine did not affect the normal social recognition capacity. This suggests that suppression of AChE activity may have distinct effects under normal and stress-induced conditions. Tg mice with higher AChE levels have accommodated themselves to this state, and it may be this accommodation that renders them incapable of facing a challenge by an anticholinesterase. One option is that of a threshold AChE-R activity that would be compatible both with satisfactory memory and normal locomotion. This balance is impaired in the Tg mice and may also be disrupted under inducers of long-term AChE-R overproduction, eg stress or exposure to anticholinesterases.[6] This, in turn, implies that the effect of anticholinesterases would depend on the initial levels of specific AChE variants in the treated mammal. Above the behaviorally-compatible threshold of AChE-R, anticholinesterases would exert behavioral improvement, whereas below it, their effects would be limited, which can explain their differential efficacy in patients with different severity of symptoms.

Glucocorticoid regulation of cholinergic behavioral patterns

The separation between general behavior patterns and learning paradigms as those relate to cholinergic transmission may explain why AChE transgenics, so dramatically impaired in their learning capacities, display such subtle deficiencies in their daily behavior. According to this concept, a constitutive hypocholinergic condition would be evident as a failure to learn and remember; however, its behavioral effect will be far less pronounced, unless challenged. This predisposition to drastic responses to external insults is indeed reminiscent of the reported behavior of demented patients. It had been initially attributed to their elevated cortisol levels,[35] which matches recent findings in primates.[36] Indeed, cortisol upregulates *ACHE* gene expression and elevates AChE-R levels,[17] possibly above the required threshold. In addition, both psychological stress and glucocorticoid hormones were reported to impair spatial working memory,[38,39] consistent with such impairments in the hAChE-S Tg mice. The intensive overexpression of mAChE-R in these mice mimics a situation in which the individual capacity for AChE-R overproduction would be tightly correlated both with the severity of the behavioral impairments induced under cholinergic hypofunction and with the capacity of anticholinesterases to affect learning and behavior properties.

Brain region specificity

Working and storage memory and the ability to integrate information are tightly linked not only to cholinergic neurotransmission, but to other neurotransmitters as well. Several studies demonstrate that even mild environmental changes (like a day-to-night switch), are accompanied by increased dopamine and noradrenaline extracellular concentration in the prefrontal cortex, and only to a minor extent in the limbic and striatal areas.[40] This activation is very selective, since molecular studies have shown that thirty minutes of restraint increase Fos protein in dopamine neurons projecting to the cortex but not in those projecting to the nucleus accumbens. In this respect altered accumbens and cortical extracellular dopamine concentrations during stress are not secondary to motor activation, but instead reflect increased attention to the provocative stimulus or attempts by the intruder to 'cope' with the stimulus, and therefore are independent of a specific motor activation.[41]

The ventral hippocampus is an important neuronal 'gate' which should be regarded as a system modulator of the cortical response to stress. In this respect cholinergic transmission may contribute to the significance of environmental cues. When neonatal ibotenic acid lesions are produced in the ventral hippocampus, repeated intraperitoneal saline injections attenuate dopamine release in the medial prefrontal cortex, while chronic haloperidol augments dopamine release in the same area of lesioned animals compared to controls.[42] This suggests that the ventral hippocampus influences the functioning of midbrain dopamine systems during environmental and pharmacological challenges in different ways.[43]

Low dose and long duration of efficacy for antisense agents

The short duration of the behavioral and memory improvements afforded by administration of tacrine parallels the time scale reported for the induction by such inhibitors of a transcriptional activation.[4] Together with a shift in alternative splicing this feedback response causes secondary AChE-R accumulation facilitating the hypocholinergic condition.[44] Recent reports demonstrate AChE accumulation in the cerebrospinal fluid of anticholinesterase-treated Alzheimer's disease patients,[45] suggesting that such feedback response occurs also in humans with cholinergic deficiencies[46] and perhaps explaining the gradual increase in anticholinesterase dosage that is necessary to maintain their palliative value in patients.

Unlike tacrine, the temporary antisense suppression of AChE synthesis improves social recognition in Tg mice for up to 6 days. This requires exceedingly low doses (25 ng per daily treatment) of the antisense agent, about 104-fold lower in molar terms than tacrine concentrations. Active site enzyme inhibitors should be administered in stoichiometric ratios with the large numbers of their protein target molecules. Moreover, the action of such inhibitors terminates when they reach their target. In contrast, a single chemically protected antisense molecule can cause the destruction of numerous mRNA transcripts, each capable of producing dozens of protein molecules. Assuming translation rates of approximately half-hour per chain and an average half-life of several hours for each transcript, destruction of each mRNA chain would prevent the production of many protein molecules. Therefore, the cumulative efficacy of antisense agents can exceed that of protein blockers by several orders of magnitude.[47,48] Moreover, the palliative effects of AS-ON destroying AChE-R mRNA should extend long after the AS-ON is destroyed, because AChE-R-induced adverse consequences would occur only above a certain threshold which takes time to accumulate. Therefore, the dose-dependent nature of the adverse consequences of AChE-R excess makes it particularly attractive as a target for antisense therapeutics.

We have recently found that AChE-R mRNA, having a long 3′ untranslated domain, is significantly more sensitive to antisense destruction than the synaptic transcript.[15,37] AChE-R mRNA transcripts would hence be preferentially destroyed, so that the excess of AChE-R, but not much of the synaptic enzyme, would decrease. This effect may explain the extended duration and increased efficacy of the antisense treatment in modifying behavior and learning exclusively in those mice with disturbed social recognition.

In conclusion, our study provides a tentative explanation for the behavioral impairments under imbalanced cholinergic neurotransmission, attributes much of these impairments to the stress-related effects of the AChE-R variant and suggests the development of antisense approach to selectively ameliorate these effects.

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References

1 Carlson NR. *Physiology of Behavior*. Allyn and Bacon: Needham Heights, MA, 1994.
2 Cummings JL, Back C. The cholinergic hypothesis of neuropsychiatric symptoms in Alzheimer's disease. *Am J Geriatr Psychiatry* 1998; 6: S64–S78.
3 Giacobini E. Present and future of Alzheimer therapy. *J Neural Transm Suppl* 2000; 59: 231–242.
4 Kaufer D, Friedman A, Seidman S, Soreq H. Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature* 1998; 393: 373–377.
5 Xie J, McCobb DP. Control of alternative splicing of potassium channels by stress hormones. *Science* 1998; 280: 443–446.
6 Kaufer D, Soreq H. Tracking cholinergic pathways from psychological and chemical stressors to variable neurodeterioration paradigms. *Curr Opin Neurol* 1998; 12: 739–743.
7 Grisaru D, Sternfeld M, Eldor A, Glick D, Soreq H. Structural roles of acetylcholinesterase variants in biology and pathology. *Eur J Biochem* 1999; 264: 672–686.
8 Sternfeld M, Shoham S, Klein R, Flores-Flores C, Evron T, Idelson GH *et al*. Excess 'readthrough' acetylcholinesterase attenuates but the 'synaptic' variant intensifies neurodeterioration correlates. *Proc Natl Acad Sci USA* 2000; 97: 8647–8652.
9 Beeri R, Andres C, Lev-Lehman E, Timberg R, Huberman T, Shani M, Soreq H. Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. *Curr Biol* 1995; 5: 1063–1071.
10 Beeri R, Le Novere N, Mervis R, Huberman T, Grauer E, Changeux JP, Soreq H. Enhanced hemicholinium binding and attenuated dendrite branching in cognitively impaired acetylcholinesterase-transgenic mice. *J Neurochem* 1997; 69: 2441–2451.
11 Erb C, Tronst J, Kopf S, Schmitt U, Loffelholz K, Soreq H, Klein J. Compensatory mechanisms enhance hippocampal acetylcholine release in transgenic mice expressing human acetylcholinesterase. *J Neurochem* 2001; 77: 638–648.

12 Shapira M, Tur-Kaspa I, Bosgraaf L, Livni N, Grauf AD, Grisaru D *et al*. A transcription-activating polymorphism in the ACHE promoter associated with acute sensitivity to anti-acetylcholinesterases. *Hum Mol Genet* 2000; 9: 1279–1281.
13 Suer C, Dolu N, Ozesmi C, Yilmaz A, Sahin O. Effect of reversed light-dark cycle on skin conductance in male rats. *Percept Mot Skills* 1998; 87: 1267–1274.
14 Thor DH, Holloway WR. Social memory of the male laboratory rat. *J Comp Psychol* 1982; 96: 1000–1006.
15 Shohami E, Kaufer D, Chen Y, Seidman S, Cohen O, Ginzberg D *et al*. Antisense prevention of neuronal damages following head injury in mice. *J Mol Med* 2000; 78: 228–236.
16 Yirmiya R, Barak O, Avitsur R, Cailliy R, Weidenfeld J, Intracerebral administration of *Mycoplasma fermentans* produces sickness behavior: role of prostaglandins. *Brain Res* 1997; 749: 71–81.
17 Stornfold M, Ming G, Song H, Sela K, Timberg R, Poo M, Soreq H. Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. *J Neurosci* 1998; 18: 1240–1249.
18 Laping NJ, Nichols NR, Day JR, Johnson SA, Finch CE. Transcriptional control of glial fibrillary acidic protein and glutamine synthetase in vivo shows opposite responses to corticosterone in the hippocampus. *Endocrinology* 1994; 135: 1828–1833.
19 Sirevaag AM, Black JE, Greenough WT. Astrocyte hypertrophy in the dentate gyrus of young male rats reflects variation of individual stress rather than group environmental complexity manipulations. *Exp Neurol* 1991; 111: 74–79.
20 McEwen BS, Sapolsky RM. Stress and cognitive function. *Curr Opin Neurobiol* 1995; 5: 205–216.
21 Hillegaart V, Ahlenius S. Time course for synchronization of spontaneous locomotor activity in the rat following reversal of the daylight (12:12 h) cycle. *Physiol Behav* 1994; 55: 73–75.
22 Winslow JT, Camacho F. Cholinergic modulation of a decrement in social investigation following repeated contacts between mice. *Psychopharmacology (Berl)* 1995; 121: 164–172.
23 Cheusi G, Bluthe RM, Goodall G, Dantzer R. Ethological study of the effects of tetrahydroaminoacridine (THA) on social recognition in rats. *Psychopharmacology (Berl)* 1994; 114: 644–650.
24 Shannon HE, Peters SC. A comparison of the effects of cholinergic and dopaminergic agents on scopolamine-induced hyperactivity in mice. *J Pharmacol Exp Ther* 1990; 256: 649–653.
25 Andres C, Beeri R, Friedman A, Lev-Lehman E, Henis S, Timberg R *et al*. Acetylcholinesterase-transgenic mice display embryonic modulations in spinal cord choline acetyltransferase and neuroxin 1B gene expression followed by late-onset neuromotor deterioration. *Proc Natl Acad Sci U S A* 1997; 94: 8173–8178.
26 Murata J, Matsukawa K, Shimizu J, Matsumoto M, Wada T, Ninomiya I. Effects of mental stress on cardiac and motor rhythms. *J Auton Nerv Syst* 1999; 75: 32–37.
27 Perio A, Termonia JP, Worms P, Bluthe RM, Dantzer R, Bizière K. Specific modulation of social memory in rats by cholinomimetic and nootropic drugs, by benzodiazepine inverse agonists, but not by psychostimulants. *Psychopharmacology* 1989; 97: 262–268.
28 Bluthe RM, Schoenen J, Dantzer R. Androgen-dependent vasopressinergic neurons are involved in social recognition in rats. *Brain Res* 1990; 519: 150–157.
29 Sakiguchi R, Wiltjorink C, van Ree JM. Short duration of retroactive facilitation of social recognition in rats. *Physiol Behav* 1991; 50: 1253–1256.
30 Soreq H, Seidman S. Acetylcholinesterase—new roles for an old actor. *Nat Rev Neurosci* 2001; 2: 294–302.
31 Fibiger HC, Damsma G, Day JC. Behavioral pharmacology and biochemistry of central cholinergic neurotransmission. *Adv Exp Med Biol* 1991; 295: 399–414.
32 Kramer TH, Buckhout R, Fox P, Widman E *et al*. Effects of stress on recall. *Appl Cog Psychol* 1991; 5: 483–488.
33 Liberzon I, Lopez JF, Flagel SB, Vazquez DM, Young EA. Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: relevance to post-traumatic stress disorder. *J Neuroendocrinol* 1999; 11: 11–17.
34 Kaneto H. Learning/memory processes under stress conditions. *Behav Brain Res* 1997; 83: 71–74.
35 Weiner MF, Vobach S, Olsson K, Svetlik D, Risser RC. Cortisol secretion and Alzheimer's disease progression. *Biol Psychiatry* 1997; 42: 1030–1038.
36 Habib KE, Weld KP, Rice KC, Pushkas J, Champoux M, Listwak S *et al*. Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. *Proc Natl Acad Sci U S A* 2000; 97: 6079–6084.
37 Grisaru D, Deutsch V, Shapira M, Pick M, Sternfold M, Melamed-Book N *et al*. ARP, a peptide derived from the stress-associated acetylcholinesterase variant, has hematopoietic growth promoting activities. *Mol Med* 2001; 7: 93–105.
38 Diamond DM, Fleshner M, Ingersoll N, Rose GM. Psychological stress impairs spatial working memory: relevance to electrophysiological studies of hippocampal function. *Behav Neurosci* 1996; 110: 661–672.
39 de Quervain DJ, Roozendaal B, McGaugh JL. Stress and glucocorticoids impair retrieval of long-term spatial memory. *Nature* 1998; 394: 787–790.
40 Conci MA, Kalen P, Mandel RJ, Bjorklund A. Regional differences in the regulation of dopamine and noradrenaline release in medial frontal cortex, nucleus accumbens and caudate-putamen: a microdialysis study in the rat. *Brain Res* 1992; 581: 217–228.
41 Cabib S, Puglisi-Allegra S. Stress, depression and the mesolimbic dopamine system. *Psychopharmacology (Berl)* 1996; 128: 331–342.
42 Deutch AY, Lee MC, Gilliham MH, Cameron DA, Goldstein M, Iadarola MJ. Stress selectively increases fos protein in dopamine neurons innervating the prefrontal cortex. *Cereb Cortex* 1991; 1: 273–292.
43 Lipska BK, Chrapusta SJ, Egan MF, Weinberger DR. Neonatal excitotoxic ventral hippocampal damage alters dopamine response to mild repeated stress and to chronic haloperidol. *Synapse* 1995; 20: 125–130.
44 Kaufer D, Friedman A, Seidman S, Soreq H. Anticholinesterases induce multigenic transcriptional feedback response suppressing cholinergic neurotransmission. *Chem Biol Interact* 1999; 119–120: 349–360.
45 Nordberg A, Hellstrom-Lindahl E, Almkvist O, Meurling L. Activity of acetylcholinesterase in CSF increases in Alzheimer's patients after treatment with tacrine. *Alzheimer's Reports* 1999; 2: 347–352.
46 Coyle JT, Price DL, DeLong MR. Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science* 1983; 219: 1184–1190.
47 Seidman S, Eckstein F, Grifman M, Soreq H. Antisense technologies have a future fighting neurodegenerative diseases. *Antisense Nucleic Acid Drug Dev* 1999; 9: 333–340.
48 Galyam N, Grisaru D, Grifman M, Melamed-Book N, Eckstein F, Seidman S *et al*. Complex host cell responses to antisense suppression of AChE gene expression. *Antisense Nucleic Acid Drug Dev* 2001; 11: 51–57.

EXHIBIT F

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Frequent Blood–Brain Barrier Disruption in the Human Cerebral Cortex

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SUMMARY

1. The blood–brain barrier (BBB) protects the brain from circulating xenobiotic agents. The pathophysiology, time span, spatial pattern, and pathophysiological consequences of BBB disruptions are not known.

2. Here, we report the quantification of BBB disruption by measuring enhancement levels in computerized tomography brain images.

3. Pathological diffuse enhancement associated with elevated albumin levels in the cerebrospinal fluid (CSF) was observed in the cerebral cortex of 28 out of 43 patients, but not in controls. Four patients displayed weeks-long focal BBB impairment. In 19 other patients, BBB disruption was significantly associated with elevated blood pressure, body temperature, serum cortisol, and stress-associated CSF "readthrough" acetylcholinesterase. Multielectrode electroencephalography revealed enhanced slow-wave activities in areas of focal BBB disruption. Thus, quantification of BBB disruption using minimally invasive procedures, demonstrated correlations with molecular, clinical, and physiological stress-associated indices.

4. These sequelae accompany a wide range of neurological disorders, suggesting that persistent, detrimental BBB disruption is considerably more frequent than previously assumed.

KEY WORDS: acetylcholinesterase; blood–brain barrier; cerebral cortex; computerized tomography.

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INTRODUCTION

The blood–brain barrier (BBB) separates the brain's interstitial space from the blood and prevents the penetrance of circulating molecules and cells into the brain (Rubin *et al.*, 1999; Soreq *et al.*, 2000). Perturbations in the integrity of the BBB have been reported in both humans (Akeson *et al.*, 1995; Cornford and Oldendorf, 1986; Klatzo, 1983; Skoog *et al.*, 1998) and animal models (Abbruscato and Davis, 1999; Friedman *et al.*, 1996) under numerous pathological conditions. In animal studies, BBB permeability can be quantitatively evaluated by measuring the concentration in the brain of nonpermeable radioactive materials, traceable macromolecules, or dyes (Abbruscato and Davis, 1999; Friedman *et al.*, 1996). However, none of these approaches is applicable to humans because of their invasiveness and the potential risks involved. Therefore, in most human studies, BBB permeability has been estimated using brain imaging techniques (computerized tomography (CT) (Roman-Goldstein *et al.*, 1994), magnetic resonance imaging (MRI) (Akeson *et al.*, 1995), or single photon emission CT (SPECT) (Siegal *et al.*, 2000)). Alternatively, altered serum constituents were searched for in the cerebrospinal fluid (CSF) (Correale *et al.*, 1998). However, no quantitative, minimally invasive approach is as yet available for evaluating BBB integrity. Therefore, the extent of enhanced BBB permeability among different patients and to different molecules, as well as the clinical correlates that predict BBB disruption await definition. Likewise, the susceptibility of different brain subregions to BBB disruption and its time resolution are still unknown. Resolution of these issues would bear wide implications to many, as it may open the way both to rationalized drug delivery into the brain and to avoidance of such penetrance when undesired. To this end, we conducted a study to develop minimally invasive means for quantifying BBB integrity. Here, we report the use of brain CT image analysis for quantitative estimation of BBB integrity in patients with various central nervous system (CNS) disorders. Our search for clinical conditions associated with compromised BBB suggests stress responses as a possible common denominator, points to the cortex as particularly vulnerable to BBB disruption, and demonstrates that such disruption may persist for at least several weeks. Focal abnormal cortical activity in areas associated with BBB disruption suggests the functional implications of such conditions. This may assist in future studies BBB integrity in various diseases and its implications to brain functioning as well as to the susceptibility to the penetrance of serum constituents, including drugs.

METHODS

Brain CT was performed using the PICKER Helical CT-TWINS (Elscint, Haifa, Israel). Scans were taken at the standard axial slices (5-mm intervals) before and ca. 1 min after the injection of the contrast agent Omnipaque (1 cm^3/kg body weight). "Control" brain scans were from randomly selected ambulatory patients who were referred for investigation, and were interpreted as "normal" by two radiologists. "Patients" CTs included patients who by signs and/or symptoms were suspected to

suffer from CNS disorders and their brain CTs interpreted as abnormal. Eighteen patients from this group, as part of their clinical investigation, were subjected to blood analysis, lumbar tap for CSF analysis and brain CT, all within 12 h. Intensity was measured bilaterally in Hounsfield units (Brooks, 1977) in round, 5–8-mm diameter regions of interest (ROIs). ROIs included subcutaneous tissue of the neck, cerebellum (2 cm lateral from the fourth ventricle), pons (one ROI at midline), thalamus (1 cm lateral from the third ventricle), white matter (at the corona radiata, bilateral to the anterior horns of the lateral ventricles), and gray matter (bilateral frontal). Using an image analysis program (Adobe PhotoShop), images (pre and post contrast agent administration) were differentiated so as to reveal contrast agent enhancement. To compare between different patients, the differential image was rescaled using a colored spectrum (blue to red) between water and bone densities (0–1000 Hounsfield Units, respectively).

MRI was performed using a Philips "Gyroscan" T5-NT machine (power track 1000, 0.5 T). SPECT scans were performed using a dual headed "Varicam" gamma camera connected to an image processing "Expert" computer (acquisition mode, format 128 × 128 pixels 120 images—each image at 3°). BBB penetration was measured following the administration of the permeable compound ^{99m}Tc-diethylenetriaminepentaacetic acid (Tc-DTPA). To measure blood perfusion to brain regions, patients were administered (more than 48 h after the Tc-DTPA study) the freely permeable compound ^{99m}Tc-ethyl cysteinate dimer (Tc-ECD) as the radioactive material. Patient clinical indices were gathered from hospital records relating to the same day when scans had been performed. When more than a single value was available for a patient, an averaged value was deduced for up to 6 h before and after time of scanning.

To detect the stress-associated readthrough variant of acetylcholinesterase (AChE-R) and its C-terminal degradation products, CSF proteins (10 μL, diluted 1:10 in PBS) were separated on 4–20% polyacrylamide gels (Bio Rad laboratories, Hercules, CA). Albumin was detected on these gels by Ponceau staining and its migration compared to that of commercially available albumin (Sigma Chemical Co., St. Louis, MO). Resultant immunoblot filters were incubated with rabbit antibodies elicited against a recombinant fusion protein of glutathione S-transferase (GST) and a peptide with the sequence of the 26 C-terminal residues of AChE-R. Antibodies were preimmunoabsorbed using GST beads to ensure specificity and were proven to interact selectively with AChE-R and not with the alternative synaptic isoform AChE-S (Sternfeld *et al.*, 2000). To detect the core AChE protein, similar blots were incubated with Chemicon anti-AChE antibodies. Detection was with peroxidase-conjugated anti-rabbit immunoglobulins and ECLTM detection (Amersham, UK).

Electroencephalogram (EEG) recordings were collected using 23 standard scalp surface electrodes (Biologic Systems Corp. Mundelein, IL) according to the 10–20 standard system with additional bilateral mastoid recordings. Electrodes impedance was kept below 5 kΩ using abrasive skin prep-cream. EEG data were collected using a CEEGRAPH IV 128-channel EEG acquisition unit (Biologic Systems Corp. Mundelein, IL) while the patient was resting supine with eyes closed. Artifact-free data from all electrodes were segmented into 2-s epochs and a power spectrum for the entire time span was calculated for each electrode.

RESULTS

Under normal conditions, the enhancement agent Omnipaque accumulates in peripheral tissues but does not significantly penetrate most CNS regions because of the functioning of the BBB. In search for impairments in BBB integrity, radiopaqueness was measured (McCullough *et al.*, 1974) before and after the injection of Omnipaque. Figure 1 demonstrates these features in a cohort of 62 patients with normal neurological examination, who were subjected to brain CT in which pathology was excluded ("controls"). Mean percent enhancement of radiopaqueness in the soft-tissue of the neck was 25.0 ± 33.9 (mean $\pm$ SEM). The large intermeasured variance is likely due to technical (amount of the injected agent, scanning latency) and/or physiological (e.g., venous return, cardiac output, or cerebral blood flow) differences. Despite this variability, linear correlation was found between signal enhancement values in the two sides of the neck tissue (Fig. 1(A)). In contrast, brain signal enhancement measured in nine different ROIs was significantly lower ($(3.4 \pm 1.8)\%$, $p < 0.0001$, t test) and displayed a much smaller variability. No correlation was found between signal enhancement in any brain region to that of the neck tissue, demonstrating bilateral impermeability of the healthy brain to Omnipaque (Fig. 1(A)).

In 43 patients with neurological signs and symptoms suspected to be due to CNS abnormality ("neurological patients"), and no focal lesion was found on bCT, mean brain enhancement ranged at 5.9–110.2% ($(12.9 \pm 15.3)\%$, mean $\pm$ SEM). This enhancement, significantly higher than that in healthy controls ($p < 0.001$, t test), suggests that in neurological patients penetrance may occur often, reflecting a frequent impairment of the BBB. In 26 patients (60%) average brain enhancement was greater than two standard deviations from the mean enhancement in the control population, reflecting significant abnormal penetrance. No significant difference was found in soft-tissue enhancement between patients and controls, exclusing the possibility that differences could be attributed to the amount of injected contrast agent or variable blood flow. In most patients, the gray and white matter of the cerebral cortex were the only regions to show larger enhancement than the control population (gray matter enhancement was 8.5 ± 13.2 and 4.5 ± 3.2 in patients and controls, respectively, $p = 0.06$, t test). Interestingly, in neurological patients percent enhancement in the left cerebral cortex showed a tendency to be higher and more variable than in the right side (10.3 ± 20.2 vs. 6.8 ± 13.1), although this was without statistical significance.

The spatial distribution of Omnipaque penetration was also analyzed in CT scans from neurological patients with high enhancement values. Figure 2 shows pseudocolored differential images from four patients with various extents and patterns of Omnipaque penetration. In normal individuals (Fig. 2(A) and data not shown), contrast material was highly concentrated and limited to venous sinuses and to brain areas that are known to lack BBB characteristics (i.e., pineal gland and neurohypophysis) (Bakay, 1976). In patients suffering from brain tumors with vasculature having abnormal BBB properties (i.e., meningiomas) (Tator and Schwartz, 1971), or following severe brain trauma, we were able to detect focal accumulation of the contrast agent in the expected location (Fig. 2(B) and data not shown). However, in certain cases, contrast agent accumulation was detected in brain tissue which

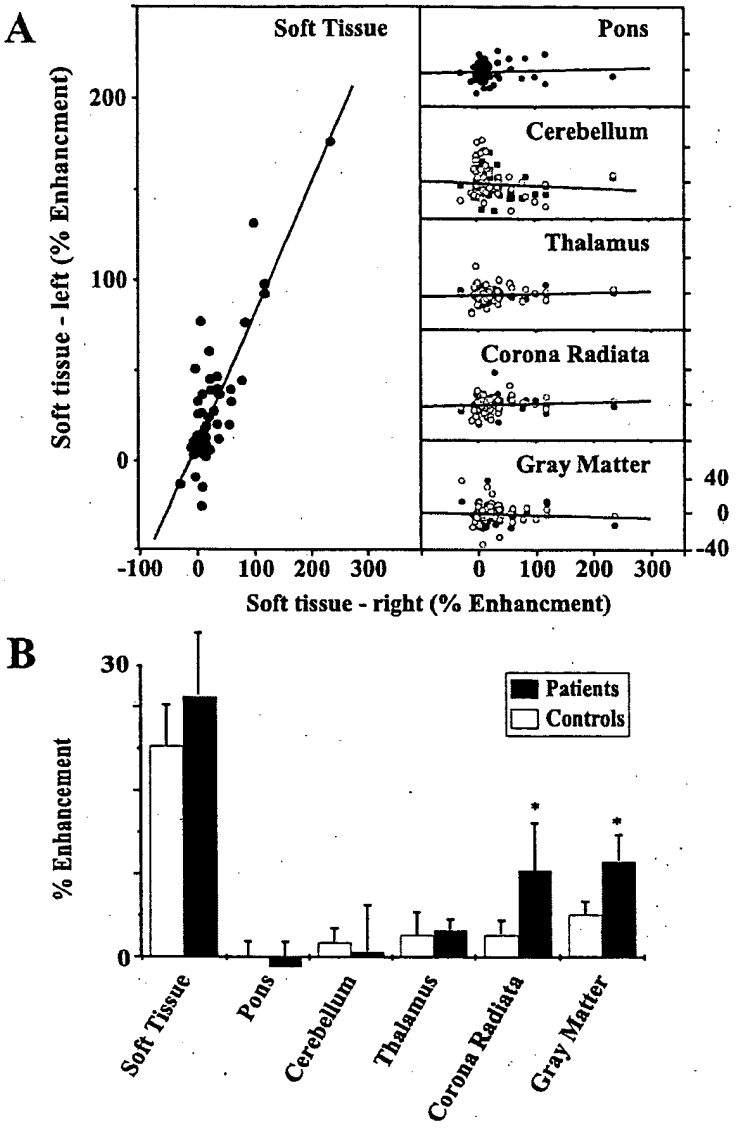

Fig. 1. CT enhancement in controls and neurological patients. (A) Control scans display impermeability to Omnipaque. All measurements were taken in Hounsfield units (HU) in 5–8-mm diameter ROIs (see Methods). Readings from the left side of the neck (soft tissue) were plotted as a function of those of the right side. Note that up to 300% bilateral enhancement was measured in neck (soft tissue, left), and no penetrance into brain regions (right). (B) Selective cortical susceptibility for BBB permeation in neurological patients. Shown are average enhancement values in controls ($n = 62$, open bars) and neurological patients ($n = 43$, filled bars). Stars note significantly higher ($p < 0.05$, t test) enhancement values in gray and white matter of the cerebral cortex (gray matter, corona radiata).

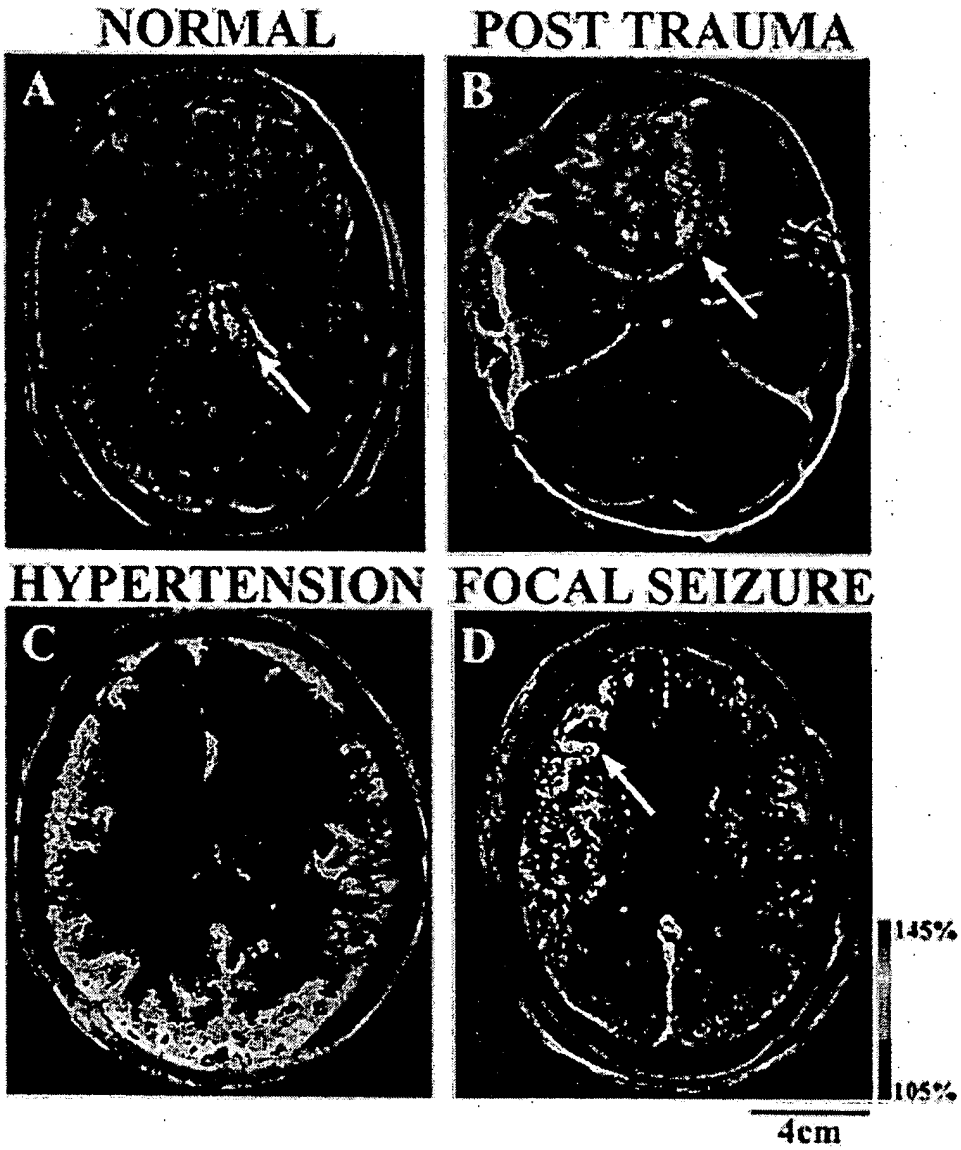

Fig. 2. Enhancement in variable brain regions in patients with CNS-related disorders. Shown are pseudocolored differential brain CT images in which signals before Omnipaque were subtracted from those following contrast agent injection. The color code refers to the range of radiopacity between water (no radiopacity, dark blue) and bone (full radiopacity, orange-red). Scale bars in all images represent 4 cm. (A) Ambulatory patient with a normal CT, with enhancement confined to choroid plexus (arrow). (B) Focal enhancement (arrow) in an 8-month-old infant 1 week following head trauma. (C) Diffuse enhancement in a 24-year-old pregnant woman presented with eclampsia. (D) Focal enhancement (arrow) in a 56-year-old man presented with a focal seizure in his left hand.

otherwise appeared normal. For example, diffuse penetration of Omnipaque into cortical gray matter was evident in a 24-year-old pregnant patient with decreased level of consciousness, who suffered from hypertensive encephalopathy (Fig. 2(C)). An abnormal focal enhancement to a cortical gyrus in the right fronto-parietal cortex was noted in another patient, presented to the emergency room with a focal seizure in his left hand (Fig. 2(D)).

Next, we wished to test the spatial distribution, persistence, and consistency of the disrupted BBB. We therefore compared brain accumulation of three different contrasting agents employed in various imaging approaches. In three patients who were subjected to several image analyses within 1 month, focal enhancement in precisely the same site was found for Omnipaque (in a CT scan), gadolinium (under MRI analysis) or Tc-DTPA (in SPECT analysis). Figure 3 shows a series of imaging studies demonstrating focal and persistently disrupted BBB in one of these patients 6 months following radiosurgery for arteriovenous malformation. One of the other two patients (images not shown) suffered from meningeal spread of tumor cells (primary CNS lymphoma) while the other from partial seizures. SPECT following administration of the brain nonpenetrating compound Tc-DTPA was then compared to perfusion analysis using the freely permeable Tc-ECD (Fig. 3(C)). In each of these three patients, focal BBB disruption was accompanied by decreased perfusion (Fig. 3(C) and data not shown). This rules out enhanced perfusion as the underlying cause for contrast agent accumulation.

The retrospective nature of our study offered an apportunity for nonbiased exploration of clinical correlates that accompany the enhancement in CT signals due to BBB disruption. To this end, we analyzed the general clinical profiles as well as the available data on serum and CSF constituents for 19 patients who were investigated within 12 h for various suspected neurological disorders but no focal abnormality was found in CT. These patients were classified by increasing order of signal enhancement and divided into three equal groups, displaying low, average, and high, diffuse gray matter enhancement of contrast agent signals (Table I). Average values of patients' blood pressure, heart rate, body temperature, white blood cell and platelet counts, CSF and serum glucose and albumin as well as serum cortisol was compared between the groups. In patients from the "low penetrance" group, the averaged postinjection signal generated by the contrast agent was not significantly different from that of preinjection ($(-1.8 \pm 3.8)\%$ enhancement, mean $\pm$ SEM), and was significantly lower than the >10% enhancement values calculated for the "high penetrance" group ($p = 0.001$). Statistically significant differences between these two groups ($p < 0.05$, Mann–Whitney test, see Table I) were also observed in body temperature, systolic and diastolic blood pressure, total CSF protein, and serum cortisol. The correlation ($r^2 = 0.85$) between enhancement values and CSF albumin concentration (Fig. 4(A)) further strengthens the notion that enhancement values indeed reflect quantitative estimations of the permeability of the BBB to large molecules. In all other clinical indices no significant difference was found between patients with low vs. high Omnipaque penetration (Table I). These findings suggest that inflammatory reactions in the peripheral or the CNS are not ultimate causes of BBB disruption (Abbott, 2000) (see Discussion).

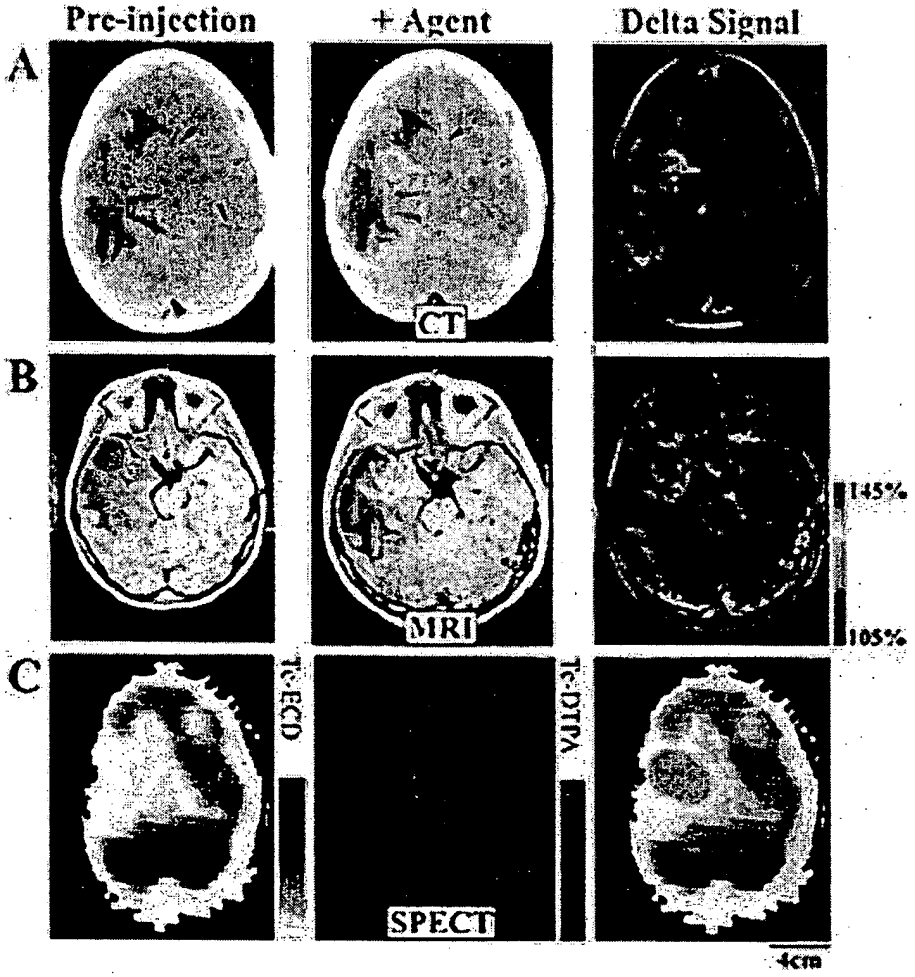

Fig. 3. BBB disruption as portrayed by CT, MRI, and SPECT. Shown are axial sections from a single patient, where BBB disruption was evident by brain signal enhancement in CT (A), MRI (B), and SPECT (C) following administration of Omnipaque, gadolinium, or Tc-DTPA, respectively, all within 1 month. Tc-ECD was used, 2 days after Tc-DTPA administration, for evaluating brain perfusion demonstrating that decreased perfusion (C, left) and increased brain penetrance (C, middle) overlapped (C, right).

BBB disruption could not be attributed to a single CNS pathology. In one patient (#16 in Table I), high CT signal enhancement was detected, yet no ovious CNS pathology found. In other patients, diagnosed for clinical conditions assumed to induce BBB disruption, no radiological or molecular evidence was found for increased BBB permeability. For example, in patients #1 and 7 who were presented with a generalized convulsion, CT displayed none or only mild cortical enhancement and no albumin was detected in their CSF. In contrast, patients #15 and 17, both of whom also presented with a generalized convulsion, displayed high penetration. Therefore, the cumulative average of our clinical and biochemical markers suggests general

Table I. Clinical Indices of "Neurological Patients"[a]

#	Age	Sex	Diagnosis	CT-% enhancement in gray matter	Hb (mg/dL)	Platelets (10^3 mm^{-3})	WBC (10^3 mm^{-3})	Serum glucose (mg/dL)	Serum cortisol (μg/dL)	CSF glucose (mg/dL)	CSF protein (mg/dL)	Heart rate (min^{-1})	B.p. systole (mbar)	B.p. diastole (mbar)	Core temp. (°C)
Low															
1	12	F	Fever, convulsions	−6.7	12.2	172	15.3	96	16.5	75	14	125	88	50	37.0
2	52	M	Coma, staphylococcal sepsis, S/P head trauma	−6.0	8.9	414	6.8	199	14.35	101	23	87	140	72	37.2
3	27	M	Headache, investigation	−2.0	15.8	340	14	132	41.7	93	27	—	—	—	—
4	1	M	Headache	0.7	13	501	15	85	16.4	66	42	118	94.5	43	37.1
5	93	M	Vomiting	1.4	11.7	215	11.7	124	26.4	81	45	89	133	64	38.2
6	19	F	Myelopathy	2.0	13.1	406	8.8	113	15.2	61	39	104	110	54	37.0
Average	**34**			**−1.8**	**12.5**	**341.3**	**11.9**	**124.8**	**21.8**	**79.5**	**21.7**	**104.6**	**113.1**	**56.6**	**37.3**
SDV	**33.6**			**3.8**	**2.2**	**126.2**	**3.5**	**40.3**	**10.7**	**15.4**	**12.2**	**16.9**	**22.9**	**11.5**	**0.5**
Medium															
7	36	F	Pneumococcal sepsis, convulsions	3.3	8.5	531	17.3	130	20.3	53	11	78	152	81	36.5
8	59	F	Fever for investigation, S/P head trauma	4.9	9.7	938	17.3	103	17.4	48	48	90	147	102	38.0
9	5/12	M	Meningitis proteus	5.4	13.7	88	8.2	70	—	3	95	140	—	—	34
10	40	F	S/P, pneumococcal meningitis	5.7	11.4	285	19.7	280	35	113	48	115	134	63	37.7
11	1	F	Febrile convulsions	5.8	12.6	288	25.9	125	—	90	14	147	113	59	37.4
12	31	M	Fever for investigation	8.2	16.8	313	10.0	111	14.8	69	41	72	120	73	37.5
13	10	M	Sagittal sinus vein thrombosis	10.1	9.6	309	10.0	103	21.1	85	16	108	120	77	37.8
Average	**25.3**			**6.2**	**11.8**	**393.1**	**15.5**	**131.7**	**21.7**	**65.9**	**39.0**	**107.1**	**131.0**	**75.8**	**36.9**
SDV	**22.1**			**2.3**	**2.9**	**272.4**	**6.4**	**68.2**	**7.3**	**35.6**	**29.5**	**29.2**	**15.9**	**15.3**	**1.4**
High															
14	68	M	Coma, investigation	11.0	11.9	193	12.3	137	28.5	97	41	114	166	92	37.7
15	1	M	Convulsive episode	12.6	11	449	10.4	70	25.6	85	26	137	115	57	37.4
16	1	M	Gastroenteritis—	15.3	7.9	669	16.1	105	27.5	—	126	148	112	55	38.3
17		M	Convulsive episode	16.7	17.3	241	10.2	393	76.2	213	64	120	143	89	37.5
18	55	F	Pneumococcal meningitis	26.4	12.3	296	20	200	92.9	62	99	103	150	72	39.2
19	69	M	Coma, investigation	33.9	10.6	497	11.4	335	20.7	184	72	112	184	88	37.6
Average	**38.8**			**19.3**	**11.8**	**390.8**	**13.4**	**206.7**	**45.2**	**128.2**	**71.3**	**122.3**	**145.0**	**75.5**	**38.0**
SDV	**34.9**			**8.9**	**3.1**	**180.3**	**3.9**	**130.5**	**31.0**	**66.2**	**36.8**	**16.9**	**28.2**	**16.6**	**0.7**
p (Mann–Whitney)	0.535			0.001	0.242	0.35	0.35	0.155	0.032	0.123	0.032	0.123	0.026	0.041	0.026

[a] Patients who were presented with "neurological signs and symptoms" but with no focal enhancing lesion in CT were evaluated. Presented in the table are the averages of available clinical data taken on several occasions within 6 h of the scan. Patients are sorted in ascending order by percent enhancement, for low, medium, and high enhancement values, averages and SDV are given in bold. In all cases the most specific available diagnosis is given. "Investigation" refers to patients who were admitted with a clinical picture indicating a neurological disorder which was not identified. "Meningitis" refers to bacterial infection (identified organism is noted). Patients #2 and 6 suffered from Head Trauma more than 1 month before the reported investigation. Patient #18 was more than 1 month following a brain surgery for total removal of meningioma. All convulsions were of the "general type." Percent enhancement in the cortical gray matter is given as the average of the two cerebral hemispheres. p values between low and high (using the nonparametric Mann–Whitney test) are presented in the last row (see text).

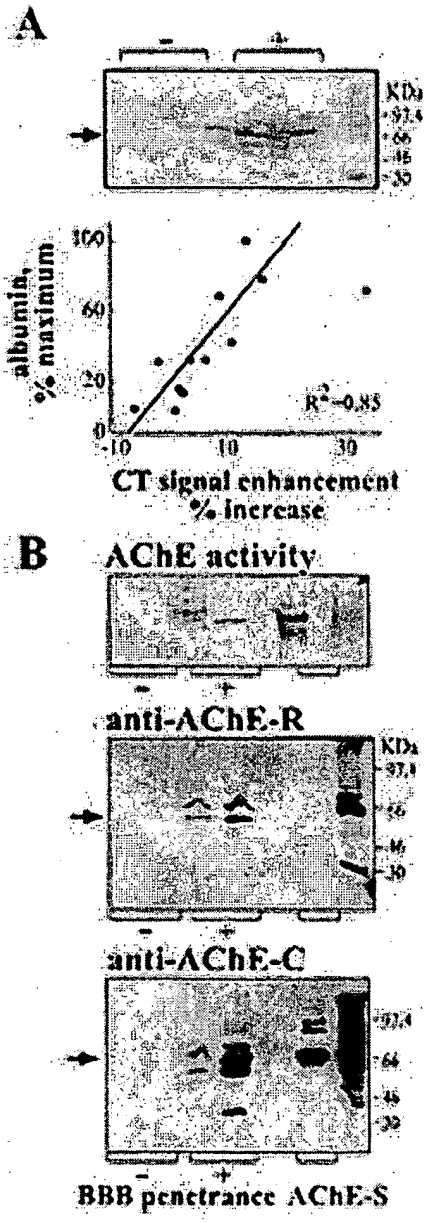

Fig. 4. BBB disruption is associated with CSF accumulation of albumin and AChE-R. (A) Albumin accumulation. Shown are albumin immunostaining following gel electrophoresis of CSF proteins (top) and correlation analysis (bottom) between CSF albumin concentrations (% of maximal level) and CT signal enhancement (% above preinjection signal intensity). Note that albumin concentrations are linearly correlated with CT signal enhancement. (B) AChE in CSF. Top: Shown is activity staining of CSF AChE following gel electrophoresis under nondenaturing conditions. Note that CSF samples with compromised (+) but not with intact (−) BBB display catalytically active AChE which co-migrates with recombinant (r) AChE-S or AChE-R produced in COS cells. Bottom: Immunoblot analyses following denaturing gel electrophoresis and incubation with antibodies selective for AChE-R (upper) or the C-terminally truncated AChE-core (lower). Note appearance of AChE-R in the same samples with AChE activity and the presence of proteolytic degradation products of the core AChE domain.

stress responses rather than a specific neurological condition as the phenotype common to patients with disrupted BBB.

Several groups have previously associated stress with focal or diffused BBB disruption (Belova and Jonsson, 1982; Ben-Nathan *et al.*, 1991; Esposito *et al.*, 2001, Friedman *et al.*, 1996; Sharma *et al.*, 1992), while others reported differently (Grauer *et al.*, 2000; Sinton *et al.*, 2000), possibly because of species, age, and experimental

manipulation differences, etc. Molecular biology approaches have associated such disruption with the rapid accumulation of the "readthrough" brain AChE-R isoform (Kaufer *et al.*, 1998a; Shohami *et al.*, 2000). To search for this AChE isoform in patients' CSF, we used antibodies selective for the C-terminal sequence unique to AChE-R. For comparison, we employed antibodies targeted to the core domain common to all AChE isoforms (Shohami *et al.*, 2000). Cytochemical staining of the electrophoretically separated CSF proteins was used to evaluate the capacity of CSF AChE to hydrolyze acetylthiocholine, a property common to all intact AChE isoforms (Soreq and Seidman, 2001). CSF samples from patients with compromised, but not with intact, BBB revealed conspicuous increases in catalytically active AChE (Fig. 4(B)). Comparative immunodetection with antibodies selective for the AChE-R variant indicated that these CSF samples included intact stress-associated AChE-R as well as degradation products from the C-terminus (Fig. 4(B)). Other AChE degradation products lacked the C-terminal peptide unique to AChE-R, as they reacted with antibodies targeted to the common core domain, but not to the C-terminus of AChE-R, and migrated faster than expected for the intact protein (Fig. 4(B)). Densitometric analysis of the immunopositive AChE-R bands displayed positive correlation with the signal enhancement levels, with an increase from an average of 29 ± 5 pixels for the low penetrance group to 58 ± 3 pixels for the high penetrance one. Further analysis will be required to test the significance of this increase in a larger group of patients.

To determine whether BBB disruption is associated with altered brain electrical activity, EEG recordings were performed on patients who showed a focal disruption in imaging studies. Figure 5 shows the results of such correlation in a 46-year-old female patient who suffered recurrent sensory disturbances in her right hand 12 months following the complete removal of a small meningioma. While MRI ruled out tumor recurrence, SPECT–DTPA showed abnormal BBB in cortical areas adjacent to the site of surgery (Fig. 5(A)). Power spectrum analyses of this patient's EEG showed clear increases in abnormal slow waves (3–6 Hz). This was associated with high alpha waves (10 Hz) activity in the records collected from the C3, but not from the C4 electrode in the contra lateral hemisphere (Fig. 5(B)). The location of these surface wave patterns matched that of a cortical area suffering from disrupted BBB (Fig. 5(B)).

DISCUSSION

In search for a minimally invasive approach for quantifying and characterizing BBB disruption in humans, we validated the use of CT brain imaging. Abnormal enhancement of brain CT signals was detected in over one half of neurological patients but not in ambulatory patients without neurological abnormalities. CSF albumin concentrations suggest that this abnormal enhancement reflects the extent of BBB disruption. Signal enhancement was also associated with significant increases in blood pressure, serum cortisol, and body temperature; in contrast, heart rate and WBC count appeared unrelated with BBB disruption. Focal BBB disruption correlated spatially with abnormal EEG, reflecting corresponding focal changes in neuronal

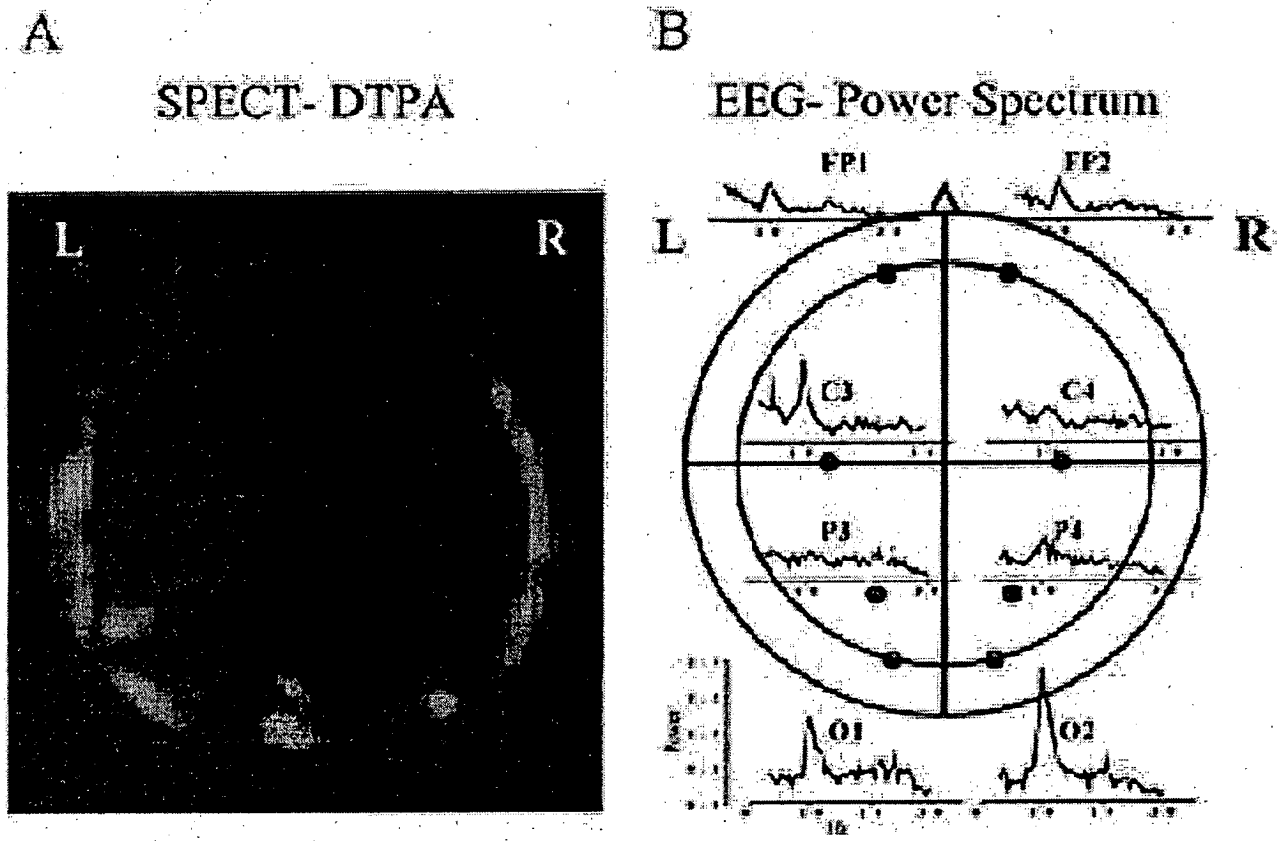

Fig. 5. EEG recordings match anatomic location of BBB disruption. (A) Axial SPECT image for the same patient performed following the administration of DTPA reveals increased signal in the left hemisphere (arrow). (B) Cortical mapping of power spectra based on 20 two-second epochs of spontaneous EEG activity (eyes closed), collected from a patient with a focal BBB disruption 12 months following surgery for the removal of a meningioma. Brain CT did not show any evidence for tumor. Spectral analyses is shown from three bilaterally matching scalp electrodes of the international 10–20 system. Left and right paired spectra are symmetrical in all electrodes, with exception of the centrally located C3 and C4 electrodes, where increased slow delta and alpha frequency are shown over the left hemisphere. This area corresponds to the area of abnormal SPECT shown in part A.

activity, as found by EEG recordings. These colonized changes indicate the importance of an intact BBB for the maintenance of normal brain function. The significant increase in CSF AChE-R accumulation in the CSF of patients with BBB disruption further emphasizes the linkage between the cholinergic system, stress, and the control over BBB permeability. Rather unexpectedly, in several patients, stable penetration patterns were observed during several weeks of clinical investigation. This suggests that BBB breakdown may persist for a long period, emphasizing the importance of the use of brain imaging for diagnosis. Recognition of BBB disruption may, in turn, call for adjustment of the treatment protocol for these patients.

The observed high incidence and persistence of BBB disruption bears both clinical and basic research implications at several levels. Regardless of the contrast agent employed, each of the brain imaging approaches yielded similar locations and apparent extent of BBB disruption. This reinforces the concept that BBB disruption may be confined to specific brain areas. The more intense general enhancement in the cerebral cortex most likely reflects a particular vulnerability of this region to BBB disruption.

The cerebral cortex microvasculature differs from other brain regions in the mechanisms controlling its blood flow (Inanami *et al.*, 1992) as well as in its cholinergic receptors' distribution (Elhusseiny *et al.*, 1999), and afferent innervation (Triguero *et al.*, 1988). This further raises the possibility that the barrier function at the level of the cerebral cortex can be modulated by neuronal activity. For example, BBB permeability has been known to increase following activation of the noreadrenergic locus coeruleus (Raichle *et al.*, 1975). Our imaging analysis supports previous reports showing BBB breakdown in epileptic patients (Cornford and Oldendorf, 1986); however, in our patients, persistent BBB disruption was observed in the absence of any evidence for active epileptic foci. Although it is generally assumed that seizures induce BBB disruption, it is not known how increased BBB permeability effects the physiological response of neurons. However, cortical neurons are known for their vulnerability to small changes in their extracellular environment (Lux, 1980; Schwartzkroin *et al.*, 1998) and for their tendency to develop synchronous epileptic activity (Jefferys, 1988). This raises the possibility of consequent neuronal toxicity (Charriaut-Marlangue *et al.*, 1996; Meldrum, 1993) in areas of persistent BBB disruption. Indeed, Kadota *et al.* (1997) reported that hippocampal neurons display excitotoxic damage following local serum infusion. Moreover, stress-induced cytokines, hormones, or small molecules such as nitric oxide (van Amsterdam and Opperhuizen, 1999) may be expected by preferentially affect cortical neurons and glial cells (Sapolsky, 1996).

At the molecular level, this study adds AChE-R to albumin as a CSF-accumulated marker of BBB disruption. Persistent stress-induced accumulation of the AChE-R protein therefore emerges as a stress response that is common to rodents and humans. The immediate source of AChE-R in human CSF can be the circulation, like albumin. Alternatively, or in addition, this secretory soluble protein may reach the CSF from stress-responding brain neurons (Shohami *et al.*, 2000) and/or endothelial cells lining vascular brain capillaries (Flumerfelt *et al.*, 1973). AChE-R degradation products may reflect the stress-induced increase in proteases (Chan and Mattson, 1999).

Apart from convulsions, with the highest predicted value for BBB disruption, our findings could only correlate this phenomenon with the intensity of stress responses. However, the mechanisms underlying stress-associated BBB disruption are yet to be found. Inflammatory responses (Rhodin *et al.*, 1999), hemodynamic changes (i.e. blood pressure) (Robinson and Moody, 1980), and brain-derived modulators are all possible candidates. Individual variations in BBB disruption may also be due to its being a complex genetic trait. For example, recent findings of signal transduction in brain endothelial cells attribute rapid and long-lasting changes in their functioning to the NO-synthase cascade (Calingasan *et al.*, 1998). Administration of NO-synthase inhibitors was indeed shown to reduce meningitis-associated BBB disruption in rodents (Boje, 1996). This calls for seeking potential correlations(s) between compromised BBB in patients and the NO pathway. Another example refers to the multiple drug resistance (*mdr1a*) gene, which encodes the drug transporting P-glycoprotein that resides in the BBB. Genomic disruption of *mdr1a* induces up to 10-fold increases in the uptake of dexamethasone into the mouse brain (Meijer *et al.*, 1998). This raises the general question whether patients under massive drug treatments, in whom the mdr1a protein is fully saturated, develop transiently modulated susceptibility for BBB disruption. A yet more specific example is that of the "atypical" allele of butyrylcholinesterase (BChE) (Loewenstein-Lichtenstein *et al.*, 1995), which increases the brain permeation of cholinesterase inhibitors due to reduced scavenging of such poisons in the circulation. Homozygous carriers of this allele may present genetic predisposition to hypersensitivity for BBB disruption under anticholinesterase exposure. Also relevant is polymorphism in a specific isoform of glutathione transferase (GSTp), which resides in endothelial cells of brain vasculature and protects the brain tissue from penetration of xenobiotics. One of the GSTp variants, with impaired substrate specificity, was found with much higher incidence in patients with Parkinson's disease, as compared to controls (Menegon *et al.*, 1998). The common denominator to all of the genes with apparent linkage to BBB disruption (mdr1a, GSTp, BCHE, and NO synthase) is that they are all involved in scavenging processes. Acquired hypersensitivity because of previous drug or chemical exposure may hence provide a major cause of BBB disruption (Kaufer *et al.*, 1998b). Increases in the risk for developing Parkinson's disease were indeed reported following exposure to brain-penetrable chemicals (e.g., lead from batteries (Kuhn *et al.*, 1998) and organophosphorous insecticides (Senanayake and Sanmuganathan, 1995)). These acquired and inherited risks may point to the intriguing possibility that BBB disruption is causally involved with neurodegenerative diseases, so that its impaired maintenance may be relevant to both acute and delayed syndromes. While our study points to stress as an important cause underlying prolonged BBB disruption in humans, future research will elucidate the association between such disruption and stress-related excitotoxic brain damage (Haley *et al.*, 2000; Sapolsky, 1996).

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REFERENCES

Abbott, N. J. (2000). Inflammatory mediators and modulation of blood-brain barrier permeability. *Cell Mol. Neurobiol.* **20:**131–147.

Abbruscato, T. J., and Davis, T. P. (1999). Protein expression of brain endothelial cell E-cadherin after hypoxia/aglycemia: Influence of astrocyte contact. *Brain Res.* **842:**277–286.

Akeson, P., Larsson, E. M., Kristoffersen, D. T., Jonsson, E., and Holtas, S. (1995). Brain metastases-comparison of gadodiamide injection-enhanced MR imaging at standard and high dose, contrast-enhanced CT and non-contrast-enhanced MR imaging. *Acta Radiol.* **36:**300–306.

Bakay, L. (1976). Blood-brain barrier and its alteration in pathological states. In Vinken, P. J., and Bruyn, G. W. (eds.), *Handbook of Clinical Neurology, Vol. 28*, Elsevier/North-Holland Biomedical Press, Amsterdam, p. 370.

Belova, I., and Jonsson, G. (1982). Blood-brain barrier permeability and immobilization stress. *Acta Physiol. Scand.* **116:**21–29.

Ben-Nathan, D., Lustig, S., and Danenberg, H. D. (1991). Stress-induced neuroinvasiveness of a neurovirulent noninvasive Sindbis virus in cold or isolation subjected mice. *Life Sci.* **48:**1493–1500.

Boje, K. M. (1996). Inhibition of nitric oxide synthase attenuates blood-brain barrier disruption during experimental meningitis. *Brain Res.* **720:**75–83.

Brooks, R. A. (1977). A quantitative theory of the Hounsfield unit and its application to dual energy scanning. *J. Comput. Assist. Tomogr.* **1:**487–493.

Calingasan, N. Y., Park, L. C., Calo, L. L., Trifiletti, R. R., Gandy, S. E., and Gibson, G. E. (1998). Induction of nitric oxide synthase and microglial responses precede selective cell death induced by chronic impairment of oxidative metabolism. *Am. J. Pathol.* **153:**599–610.

Chan, S. L., and Mattson, M. P. (1999). Caspase and calpain substrates: Roles in synaptic plasticity and cell death. *J. Neurosci. Res.* **58:**167–190.

Charriaut-Marlangue, C., Aggoun-Zouaoui, D., Represa, A., and Ben-Ari, Y. (1996). Apoptotic features of selective neuronal death in ischemia, epilepsy and gp 120 toxicity. *Trends Neurosci.* **19:**109–114.

Cornford, E. M., and Oldendorf, W. H. (1986). Epilepsy and the blood-brain barrier. *Adv. Neurol.* **44:**787–812.

Correale, J., Rabinowicz, A. L., Heck, C. N., Smith, T. D., Loskota, W. J., and DeGiorgio, C. M. (1998). Status epilepticus increases CSF levels of neuron-specific enolase and alters the blood-brain barrier. *Neurology* **50:**1388–1391.

Elhusseiny, A., Cohen, Z., Olivier, A., Stanimirovic, D. B., and Hamel, E. (1999). Functional acetylcholine muscarinic receptor subtypes in human brain microcirculation: Identification and cellular localization. *J. Cereb. Blood Flow Metab.* **19:**794–802.

Esposito, P., Gheorghe, D., Kandere, K., Pang, X., Connolly, R., Jacobson, S., and Theoharides, T. C. (2001). Acute stress increases permeability of the blood-brain barrier through activation of brain mast cells. *Brain Res.* **888:**117–127.

Flumerfelt, B. A., Lewis, P. R., and Gwyn, D. G. (1973). Cholinesterase activity of capillaries in the rat brain. A light and electron microscopic study. *Histochem. J.* **5:**67–77.

Friedman, A., Kaufer, D., Shemer, J., Hendler, I., Soreq, H., and Tur-Kaspa, I. (1996). Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. *Nat. Med.* **2:**1382–1385.

Grauer, E., Alkalai, D., Kapon, J., Cohen, G., and Raveh, L. (2000). Stress does not enable pyridostigmine to inhibit brain cholinesterase after parenteral administration. *Toxicol. Appl. Pharmacol.* **164:**301–304.

Haley, R. W., Marshall, W. W., McDonald, G. G., Daugherty, M. A., Petty, F., and Fleckenstein, J. L. (2000). Brain abnormalities in Gulf War syndrome: Evaluation with 1H MR spectroscopy. *Radiology* **215:**807–817.

Inanami, O., Ohno, K., and Sato, A. (1992). Responses of regional cerebral blood flow to intravenous administration of thyrotropin releasing hormone in aged rats. *Neurosci. Lett.* **143:**151–154.

Jefferys, J. G. (1998). Mechanisms and experimental models of seizure generation. *Curr. Opin. Neurol.* **11:**123–127.

Kadota, E., Nonaka, K., Karasuno, M., Nishi, K., Teramura, K., and Hashimoto, S. (1997). Neurotoxicity of serum components, comparison between CA1 and striatum. *Acta Neurochir. Suppl. (Wien)* **70**:141–143.

Kaufer, D., Friedman, A., Seidman, S., and Soreq, H. (1998a). Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature* **393**:373–377.

Kaufer, D., Friedman, A. Seidman, S., and Soreq, H. (1998b). Anticholinesterases induce multigenic transcriptional feedback response suppressing cholinergic neurotransmission. *Chem. Biol. Interact.* **119**:349–360.

Klatzo, I. (1983). Disturbance of the blood-brain barrier in cerebrovascular disorders. *Acta Neuropathol. Suppl. (Berl)* **8**:81–8.

Kuhn, W., Winkel, R., Woitalla, D., Meves, S., Przuntek, H., and Muller, T. (1998). High prevalence of parkinsonism after occupational exposure to lead-sulfate batteries. *Neurology* **50**:1885–1886.

Loewenstein-Lichtenstein, Y., Schwarz, M., Glick, D., Norgaard-Pedersen, B., Zakut, H., and Soreq, H. (1995). Genetic predisposition to adverse consequences of anti-cholinesterases in 'atypical' BCHE carriers. *Nat. Med.* **1**:1082–1085.

Lux, H. D. (1980). Ionic conditions and membrane behavior. *Adv. Neurol.* **27**:63–83.

McCullough, E. C., Baker, H. L., Jr., Houser, O. W., and Reese, D. F. (1974). An evaluation of the quantitative and radiation features of a scanning x-ray transverse axial tomograph: The EMI scanner. *Radiology* **111**:709–715.

Meijer, O. C., de Lange, E. C., Breimer, D. D., de Boer, A. G., Workel, J. O., and de Kloet, E. R. (1998). Penetration of dexamethasone into brain glucocorticoid targets is enhanced in mdr1A P-glycoprotein knockout mice. *Endocrinology* **139**:1789–1793.

Meldrum, B. S. (1993). Excitotoxicity and selective neuronal loss in epilepsy. *Brain Pathol.* **3**:405–412.

Menegon, A., Board, P. G., Blackburn, A. C., Mellick, G. D., and Le Couteur, D. G. (1998). Parkinson's disease, pesticides, and glutathione transferase polymorphisms. *Lancet* **352**:1344–1346.

Raichle, M. E., Hartman, B. K., Eichling, J. O., and Sharpe, L. G. (1975). Central noradrenergic regulation of cerebral blood flow and vascular permeability. *Proc. Natl. Acad. Sci. U.S.A.* **72**:3726–3730.

Rhodin, J., Thomas, T., Bryant, M., Clark, L., and Sutton, E. T. (1999). Animal model of vascular inflammation. *J. Submicrosc. Cytol. Pathol.* **31**:305–311.

Robinson, J. S., and Moody, R. A. (1980). Influence of respiratory stress and hypertension upon the blood-brain barrier. *J. Neurosurg.* **53**:666–673.

Roman-Goldstein, S., Clunie, D. A., Stevens, J., Hogan, R., Monard, J., Ramsey, F., and Neuwelt, E. A. (1994). Osmotic blood-brain barrier disruption: CT and radionuclide imaging. *AJNR Am. J. Neuroradiol.* **15**:581–590.

Rubin, L. L., and Staddon, J. M. (1999). The cell biology of the blood-brain barrier. *Annu. Rev. Neurosci.* **22**:11–28.

Sapolsky, R. M. (1996). Why stress is bad for your brain. *Science* **273**:749–750.

Schwartzkroin, P. A., Baraban, S. C., and Hochman, D. W. (1998). Osmolarity, ionic flux, and changes in brain excitability. *Epilepsy Res.* **32**:275–285.

Senanayake, N., and Sanmuganathan, P. S. (1995). Extrapyramidal manifestations complicating organophosphorus insecticide poisoning. *Hum. Exp. Toxicol.* **14**:600–604.

Sharma, H. S., Kretzschmar, R., Cervos-Navarro, J., Ermisch, A., Ruhle, H. J., and Dey, P. K. (1992). Age-related pathophysiology of the blood-brain barrier in heat stress. *Prog. Brain Res.* **91**: 189–196.

Shohami, E., Kaufer, D., Chen, Y., Seidman, S., Cohen, O., Ginzberg, D., Melamed-Book, N., Yirmiya, R., and Soreq, H. (2000). Antisense prevention of neuronal damages following head injury in mice. *J. Mol. Med.* **78**:228–236.

Siegal, T., Rubinstein, R., Bokstein, F., Schwartz, A., Lossos, A., Shalom, E., Chisin, R., and Gomori, J. M. (2000). In vivo assessment of the window of barrier opening after osmotic blood-brain barrier disruption in humans. *J. Neurosurg.* **92**:599–605.

Sinton, C. M., Fitch, T. E., Petty, F., and Haley, R. W. (2000). Stressful manipulations that elevate corticosterone reduce blood-brain barrier permeability to pyridostigmine in the rat. *Toxicol. Appl. Pharmacol.* **165**:99–105.

Skoog, I., Wallin A., Fredman, P., Hesse, C., Aevarsson, O., Karlsson, I., Gottfries, C. G., and Blennow, K. (1998). A population study on blood-brain barrier function in 85-year-olds: Relation to Alzheimer's disease and vascular dementia. *Neurology* **50**:966–971.

Soreq, H., Kaufer, D., Friedman, A., and Glick, D. (2000). Blood-brain barrier modulations and low-level exposure to xenobiotics. In Somani S. M., and Romano J. A. (eds.), *Chemical Warefare Agents: Low Level Toxicity*, CRC Press, Boca, Raton, FL, pp. 121–144.

Soreq, H., and Seidman, S. (2001). Acetylcholinesterase—new roles for an old actor. Nature *Neurosci. Rev.* **2**:294–302.

Sternfeld, M., Shoham, S., Klein, O., Flores-Flores, C., Evron, T., Idelson, G. H., Kitsberg, D., Patrick, J. W., and Soreq, H. (2000). Excess "read-through" acetylcholinesterase attenuates but the "synaptic" variant intensifies neurodeterioration correlates. *Proc. Natl. Acad. Sci. U.S.A.* **97**:8647–8652.

Tator, C. H., and Schwartz, M. L. (1971). Permeability in brain tumors. *J. Neurosurg.* **34**:460–462.

Triguero, D., Lopez de Pablo, A. L., Gomez, B., and Estrada, C. (1988). Regional differences in cerebrovascular cholinergic innervation in goats. *Stroke* **19**:736–740.

van Amsterdam, J. G., and Opperhuizen, A. (1999). Nitric oxide and biopterin in depression and stress. *Psychiatry Res.* **85**:33–38.

EXHIBIT G

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ORIGINAL ARTICLE

Endotoxin-Induced Changes in Human Working and Declarative Memory Associate with Cleavage of Plasma "Readthrough" Acetylcholinesterase

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Abstract

Endotoxin stimulation of the immune system produces marked alterations in memory functioning. However, molecular links between this cognitive response and infection-responding neurotransmission pathways are still unknown. The cytokine and memory responses of volunteers injected with 0.8 ng/kg *Salmonella* endotoxin were compared with changes in plasma levels and integrity of the stress-induced acetylcholinesterase variant, AChE-R. Vascular endothelial cells were found to express AChE-R messenger RNA and protein both in healthy and inflamed human tissues. Plasma AChE activity was reduced after endotoxin treatment, but not placebo treatment, parallel to the decline in cortisol after the endotoxin-induced peak and inversely to the accumulation of a C-terminal immunopositive AChE-R peptide of 36 amino acid residues. AChE-R cleavage coincided with significant endotoxin-induced improvement in working memory and impairment in declarative memory. By 3 h posttreatment, working memory improvement was negatively correlated with AChE-R cleavage, which showed association to proinflammatory cytokine levels. By 9 h posttreatment, declarative memory impairment was negatively correlated with AChE-R cleavage and positively correlated with the suppressed AChE activity. Endotoxin-induced peripheral cholinergic stress responses are associated with greater impairment in declarative memory and lower improvement in working memory, pointing at AChE-R as a surrogate marker of psychoneuroimmunological stress.

Index Entries: Acetylcholinesterase; cortisol; cytokines; endotoxin; inflammation; declarative memory; working memory.

Introduction

Numerous medical conditions involve memory disturbances (e.g., Alzheimer's disease (Arendt 2001), multiple sclerosis (Thornton 2002), acquired immunodeficiency syndrome (Navia 1986), and infectious diseases (Capuron 1999). Parallel inflammatory responses and production of cytokines, particularly within the brain, raised the suggestion that illness-associated alterations in memory functioning are at least partly mediated by immune activation (Yirmiya, 1997; Maier and Watkins, 1998; Rachal Pugh, 2001). Cytokine-induced memory impairments in humans, including cancer and hepatitis C patients (Meyers, 1999; Capuron, 2001), as well as in experimental animals (Oitzl, 1993; Gibertini, 1996),

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support this notion. Thus, as with many other stressful stimuli that are known to affect learning and memory processes (Kim and Diamond, 2002), inflammation can cause marked alterations in memory functioning.

Administration of endotoxin (lipopolysaccharide), a complex glycolipid found in the outer membrane of all Gram-negative bacteria, serves to assess the cognitive consequences of the acute host response to infection in humans. Endotoxin administration induces fever, malaise, and increased production and secretion of cytokines, particularly tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1 and IL-1ra, and cortisol (for review *see* Burrell, 1994), as well as proteases (Fahmi and Chaby, 1994). In healthy humans, endotoxin-induced cytokine secretion is correlated with impairments in verbal and nonverbal declarative memory functions (Reichenberg, 2001).

Memory deficits and profound neurobehavioral and neuroendocrine symptoms were also reported to be correlated with endotoxin-induced secretion of cytokines in experimental animals (Pugh, 1998; Hauss-Wegrzyniak, 2000; Shaw, 2001). Although these findings suggest that cytokines are involved in mediating the effects of endotoxin on memory, little is known about the neurotransmission pathways associated with these cytokine activities. We initiated a search into the possibility that cholinergic processes are relevant to endotoxin responses because, in the central nervous system, cholinergic responses are notably involved in several important aspects of cognitive functioning, including attention, learning, and memory (for reviews, *see* Segal and Auerbach, 1997; Levin and Simon, 1998). Moreover, endotoxin decreases brain choline acetyltransferase activity (Willard, 1999), similar to the effects of psychological stress (Kaufer, 1998). In the periphery, endogenous or exogenous acetylcholine (ACh) attenuates the release of proinflammatory cytokines from endotoxin-stimulated human macrophages (Tracey, 2001; Borovikova, 2000; Bernik, 2002). The ACh-hydrolyzing enzyme acetylcholinesterase (AChE) was considered potentially being of particular relevance to these processes because AChE controls ACh levels and because AChE inhibitors improve cognitive functions in both clinical and experimental paradigms (Weinstock, 1995; Palmer, 2002). Moreover, AChE overexpression is triggered by acute and chronic stressful insults (Meshorer, 2002) and induces progressive memory impairments, as was demonstrated in transgenic mice (Beeri, 1995).

Stress-induced transcriptional activation of AChE gene expression is associated with accumulation of the normally rare "readthrough" AChE-R splice variant (Soreq and Seidman, 2001). In the short range, the AChE-R excess reduces the stress-induced cholinergic hyperexcitation (Kaufer, 1998); in the long range, it induces hypersensitivity to cholinergic agonists and antagonists (Meshorer, 2002) and, when inherited, limits stress-induced neuropathologies (Sternfeld, 2000). Mice that overexpress both AChE-S and AChE-R present progressive dendritic and spine loss (Beeri, 1997), as well as altered anxiety responses (Erb, 2001). Furthermore, these mice display early-onset deficits in social recognition and exaggerated responsiveness to stressful insults. These can be briefly ameliorated by conventional anticholinesterase treatment or for longer periods by an antisense oligonucleotide capable of specifically inducing the destruction of AChE-R messenger RNA (mRNA) (Cohen, 2002), suggesting that AChE-R is the primary cause. Thus, AChE-R production may lead to both positive and negative effects on cognition.

The role of cholinergic mechanisms in learning and memory, the involvement of AChE-R in stress responses, the suppression by ACh of proinflammatory cytokines production, and the effects of endotoxin on memory functions suggested involvement of AChE-R in mediating endotoxin-induced memory alterations. Stressful insults induce AChE-R production in the periphery as well (e.g., in the small intestines), and failure to induce this production in response to aversive stimuli results in hypersensitivity to relatively mild stressors (Shapira, 2000). This raised the possibility that peripheral AChE modulations may serve as a surrogate marker of endotoxin-induced changes in cognition as well. However, in plasma, proteolytic cleavage of AChE-R leads to the appearance in the serum of a short immunopositive C- terminal peptide, which facilitates the hematopoietic stress responses (Grisaru, 2001). Hence, we investigated the effects of endotoxin administration on both AChE activity and AChE-R cleavage in healthy human volunteers and explored potential correlations between these parameters, the secretion of cytokines or cortisol, and changes with time in memory functions. In addition to declarative memory, which involves consciously accessible records of facts and events through concerted functioning of hippocampal and prefrontal structures (Kim and Diamond, 2002), we also assessed the effects of endotoxin and its interactions

with AChE cleavage on working memory, which involves temporary storage and manipulation of information necessary for cognitive functioning (Baddeley, 1992) and has been shown to involve prefrontal cholinergic mechanisms (Furey, 2000).

Methods

Subjects

Ten male subjects participated in the study, which was approved by an independent ethics committee. Subjects recruitment as well as physical and psychiatric screening were described in detail elsewhere (Reichenberg, 2001). The current study involved a subset of the subjects included in the previous project, with serum AChE and working memory tests added. Interviews by experienced psychiatrists excluded the presence and the history of any axis I psychiatric disorder according to the Diagnostic and Statistical Manual for Mental Disorders–IV (American Psychiatric Association, 1994). Only subjects who successfully passed the screening procedure, and signed an informed consent form were considered eligible to participate. Comprehensive assessment was performed and involved each subject going through a number of physical and neuropsychological tests in a clinical research unit using a balanced, randomized, double-blind, crossover design.

Procedure

All technical equipment, including the blood sampling device, was housed in a room adjacent to the sound-shielded experimental room. Every subject passed two 10-d-apart testing sessions and spent the night before each experimental session in the research unit. A battery of neuropsychological tests, assessing memory, learning, and attention was given for adaptation upon their first arrival in the evening, minimizing subsequent practice effects (McCaffrey and Lynch, 1992). Alternate versions of these tests were used in the experimental testing sessions. The next morning, an intravenous (iv) cannula was inserted into an antecubital forearm vein for intermittent blood sampling and iv injection of endotoxin (0.8 ng *Salmonella abortus equi* endotoxin per kilogram of body weight) in one session or the same volume of 0.9% NaCl (saline) solution on the other occasion (placebo). The order of injections was balanced, so that half of the subjects received the saline injection and half received the endotoxin injection first. No significant differences were found between the groups defined by the treatment order in either age, years of education, or body weight. The experimenter and the subject were blind with respect to the group assignment. During each session, subjects were tested three times, at 1–2, 3–4, and 9–10 h post-injection. Blood was collected at baseline before iv injection and at the beginning of each testing period. Rectal temperature was measured continuously using a thermistor probe. Self-reported physical sickness symptoms (headaches, muscle pain, shivering, nausea, breathing difficulties, and fatigue) were assessed at the end of each testing period, by a questionnaire using a 5-point Likert scale (0 = no symptoms, 4 = very severe symptoms).

Salmonella abortus equi *Endotoxin*

Prepared for use in humans, this endotoxin was available as a sterile solution free of proteins and nucleic acids. The endotoxin preparation employed has proven to be safe in various studies of other groups (for review, *see* Burrell, 1994)) and in studies at the Max Planck Institute of Psychiatry, including more than 100 subjects since 1991 (Pollmacher, 1996).

Plasma Levels of AChE and Its Degradation Product, Cytokines, and Cortisol

Blood was collected in tubes containing Na-EDTA and aprotinin and was immediately centrifuged. Plasma was aliquoted and frozen to –80°C. AChE catalytic activity was measured as the capacity for acetylthiocholine (ATCh) hydrolysis in the presence of 1×10^{-5} *M* tetraisopropylpyrophosphoramidate (iso-OMPA), a selective inhibitor of serum butyrylcholinesterase (BChE; Soreq and Glick, 2000). Endotoxin-induced differences were calculated by subtracting activities in the absence of endotoxin, with each individual serving as its own control and daily hour carefully matched. To evaluate AChE-R concentrations and integrity, plasma proteins (40 µg) were subjected to 4–20% polyacrylamide gel electrophoresis under fully denaturating conditions (Bio-Rad Laboratories, Hercules, CA), blotted to nitrocellulose filters, incubated with rabbit anti–AChE-R antibodies (Sternfeld, 2000) and peroxidase-conjugated anti-rabbit immunoglobulins, and subjected to ECL detection (Amersham Pharmacia Biotech, UK), densitometric analysis, and quantification as described (Shohami, 2000). The plasma levels of cortisol were determined by a radioimmunoassay, and the plasma levels of cytokines and soluble cytokine receptors were assessed by commercial enzyme-linked immunosorbent assays (for more details *see* Mullington, 2000).

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Labeling AChE-R mRNA and Its Protein Product in Vascular Endothelial Cells

Fluorescent *in situ* hybridization and immunohistochemistry of AChE-R mRNA and AChE-R protein were performed and quantified as reported (Cohen, 2002; Perry, 2002) using paraffin-embedded tissue sections from surgically removed biopsies of patients with or without clinical inflammation resulting from nonspecific kidney vasculitis or after kidney rejection.

MALDI-TOF-MS Analysis of Immunolabeled Proteins

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was employed in an attempt to identify the protein and peptide bands labeled by anti-AChE-R antibodies in blotted membranes. Proteolytic degradation of the gel-eluted peptide was performed using the endoprotease LysC from Achromobacterlyticus (Wako Chemicals, Inc., USA) at a substrate to enzyme ratio of 200:1. Digestion was carried out overnight in 0.05 *M* Tris-HCl, pH 9.0, containing 4 *M* urea at 30°C.

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Neuropsychological Assessment

Declarative memory was assessed using the *Story Recall* test (Green and Allen, 1995): subjects were requested to repeat a 25-item story from memory immediately, and 30 min after presentation. The total number of correct verbatim recall was counted. Memory span and working memory were assessed using the *Digit Span Forward & Backward* (Wechsler, 1987): subjects were requested to repeat lists of digits with increased number of digits every two lists either in the correct order of presentation (*forward condition*, assessment of span) or in a reversed order (*backward condition*, assessment of working memory). The number of lists correctly repeated was counted. Attention was assessed using the Ruff 2 & 7 cancellation test (Ruff and Allen, 1996): Subjects were instructed to mark either the digit 2 or the digit 7, which are randomly placed either between letters or between digits. The numbers of correct responses in a 5-min trial were counted.

Statistical Analyses

The main hypotheses concerning treatment effects on AChE activity, AChE-R levels, and neuropsychological performance were tested using repeated measure analysis of variance models (ANOVAs). Repeated measure ANOVAs were also used to examine the treatment effect on physical sickness symptoms, on plasma levels of cytokines and cortisol and on body temperature. The level of significance was set at the critical value of $p <= 0.05$ (two-tailed). Whenever significant treatment-by-time interactions were found, the simple effects were analyzed as suggested (Winer, 1991), and Tukey's adjustments were applied.

To assess the associations between changes from the placebo to the endotoxin condition in AChE activity, AChE-R levels, and physiological (cytokines and cortisol secretion), and neuropsychological parameters, Pearson's correlation coefficients were calculated.

No deviation from normal distributions was evident for any of the dependent variables. No univariate outliers were found using Z-scores and no multivariate outliers were found using the Mahalanobis distance (Tabachnick and Fidell, 2001). To adjust for any nonhomogeneity of covariance for the within-subject effects, we used p values that were adjusted using the Huynh-Feldt method (Norusis, 1994). Analyses were carried out using SPSS 10.

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Results

Endotoxin Induces Impairments in AChE-R Activity and Integrity

Endotoxin administration produced a time-dependent decrease in plasma AChE activity, measured by quantifying the rate of ATCh hydrolysis in the presence of the butyrylcholinesterase (BChE) inhibitor iso-OMPA. This reduction displayed a significant treatment-by-time interaction (Fig. 1A), ($F(2,16) = 3.94$, $p <= 0.04$). Saline administration (placebo) caused no change in AChE activity, excluding the possibilities that it was induced by the injection stress or by circadian influences. The decline in hydrolytic activity could potentially reflect losses in the AChE protein. To test this possibility, electrophoretically separated plasma proteins were immune-reacted with antibodies selective for the C-terminal peptide unique to AChE-R (Sternfeld, 2000). These antibodies labeled a 66-kd protein, likely to be full-length AChE-R, as well as a shorter peptide with an apparent size of 6.5kd. A parallel labeling pattern in the serum of stressed mice (Grisaru, 2001) raised the suggestion that this was an immunopositive C-terminus cleavage product of AChE-R. Endotoxin administration induced a slight, yet persistent, increase in the AChE-R cleavage product (Fig. 1 B,C). This increase did not reach statistical significance [$F(1,8) = 2.32$, $p <= 0.16$, for treatment

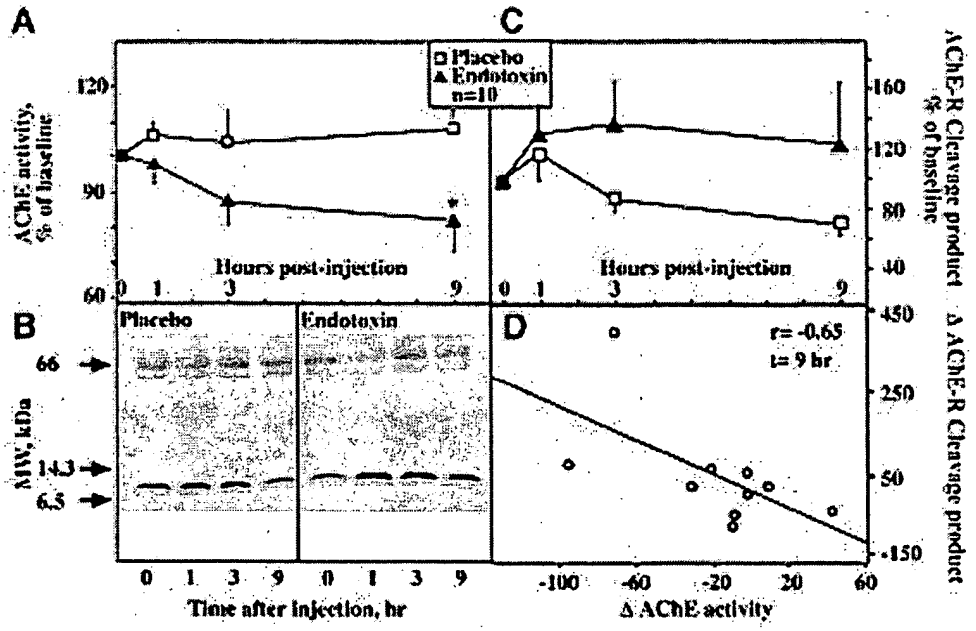

Fig. 1. Changes over time in the plasma levels of AChE activity and in AChE-R cleavage. (A) Hydrolytic activities. Shown are plasma AChE activities (mean ± SEM) for 10 volunteers injected twice, with endotoxin or saline (placebo) at the noted intervals after injection. Preinjection (baseline) AChE level was considered as 100% for each individual. Asterisks denote statistical difference ($p < 0.05$). (B) Immunoblot. Shown are consecutive results for one individual. Plasma samples were electrophoresed by SDS-PAGE, and the blot immunoreacted with anti-AChE-R antibodies. Note the 6.5 kDa AChE-R cleavage product. Left lanes indicate the response to a placebo injection; right lanes demonstrate elevated AChE-R cleavage in response to endotoxin. (C) Densitometric intensities. Shown are average values (mean ± S.E.M.) of the rapidly migrating AChE-R cleavage product in plasma of the endotoxin and placebo treated individuals (Methods) as % of baseline (described in A). Note: Elevated AChE-R cleavage in endotoxin-treated subjects coappeared with decreased AChE activity. (D) Association analysis. Highly significant negative association (correlation coefficient, $r = -0.65$) emerged between the increases in AChE-R cleavage and the decrease in AChE activity under endotoxin during the last testing period ($t = 9$ h). Each dot represents a single individual.

effect] (Fig. 1C). However, at 9 h posttreatment, the endotoxin-induced decrease in AChE activity was significantly correlated with endotoxin-induced increase in AChE-R cleavage ($r = -0.65$) (Fig. 1D).

MALDI-TOF-MS Analysis of AChE-R Cleavage Product

To further characterize the AChE-R cleavage product, larger plasma samples (180 µg/lane) were resolved by electrophoresis. Protein bands that comigrated with the bands labeled with anti–AChE-R antibodies were cut out of the gel and subjected to MALDI-TOF-MS analyses. The elution product of the larger band was identified as being mainly composed of serum albumin (molecular weight, 69367), compatible with the assumption that AChE-R is only a minor component in this size fraction of human serum proteins. The shorter peptide eluted from the excised band, however, revealed a single peak with a molecular mass of 3613–3615. Figure 2 demonstrates the MALDI-TOF-MS profile of this eluted peptide. Peptide property calculations positioned the presumed proteolytic cleavage site 36 residues from the C-terminus of AChE-R, with a calculated mass of 3614. Under these assumptions, cleavage could occur between asparagine and arginine residues upstream to the AChE-R diversion site (Fig. 2).

Parallel size peptides were observed in gel-eluted products from several individuals, demonstrating consistent cleavage processes. LysC proteolysis failed to further shorten this peptide. Edman degradation was unsuccessful, perhaps because of N-terminal blockade, and further experiments were prevented because of lack of material. The mass spectrometry approach thus pointed, although inconclusively, at an AChE-R cleavage site in human

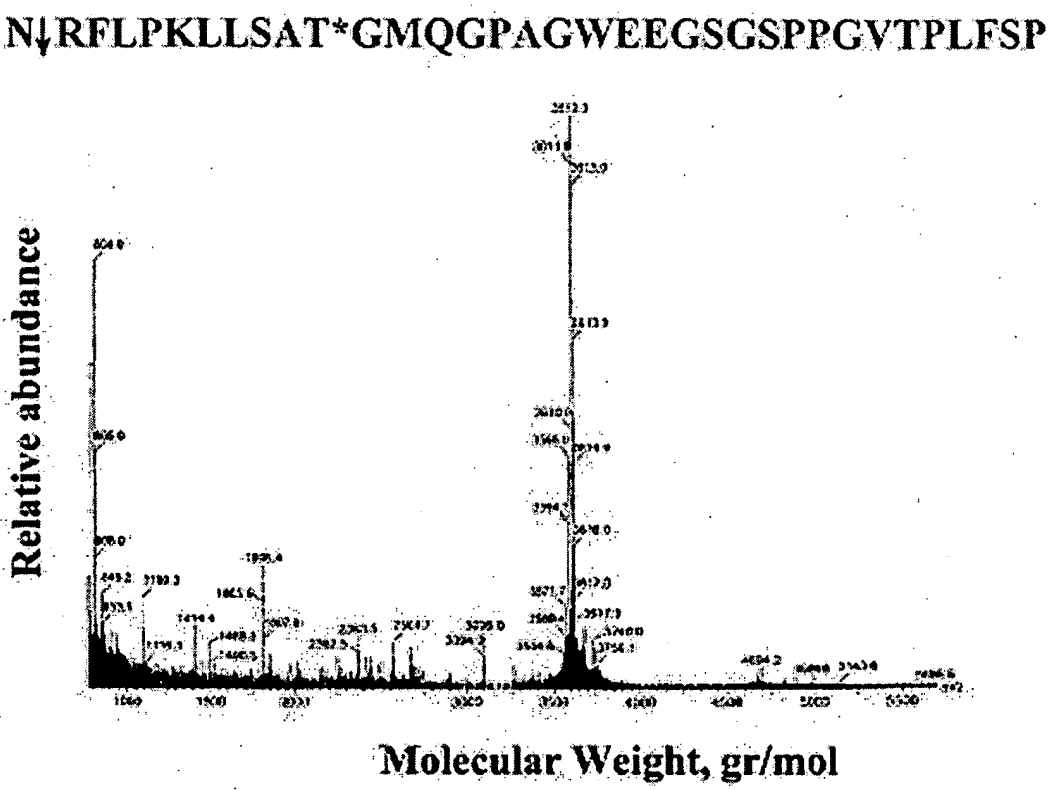

Fig. 2. Mass spectroscopy of gel-eluted band. Shown is the outcome of electron spray mass spectrometry analysis of the gel-eluted rapidly migrating band that immunoreacted with anti-AChE-R antibodies (Methods). Note that the main peptide displayed a molecular mass of 3613–3615. Calculation of predicted masses positioned the presumed proteolytic cleavage site 36 residues from the C-terminus of AChE-R, between asparagine and arginine residues in the sequence presented, with the presumed cleavage site arrowed and the diversion site starred.

plasma under endotoxic stress near the C-terminal splice site that marks the deviation between human AChE splice variants.

Vascular Endothelial Cells Produce AChE-R

In search for the potential cell type origin of plasma AChE-R, we performed fluorescent in situ hybridization (FISH) and immunohistochemistry on human tissues from patients with or without inflammatory diseases (e.g., kidney vasculitis). Vascular endothelial cells displayed labeling with both AChE-R complementary RNA and anti-AChE-R antibodies (Fig. 3A,B). Quantification of signal intensities revealed considerable similarities between AChE-R mRNA and AChE-R protein levels in patients with or without inflammatory vasculitis, so that tissues with less pronounced mRNA labeling also displayed fainter protein labeling (Fig. 3C). This pointed at vascular endothelial cells, which also harbor non-neuronal nicotinic acetylcholine receptors (Heeschen, 2002) as a probable site of continuous plasma AChE-R production.

AChE-R Cleavage Is Associated with Cytokines Secretion

Endotoxin induced a transient, significant increase in the plasma levels of cortisol, TNF-α, and IL-6 (Fig. 4A–C), but does not produce any significant effects on the subjective rating of physical or behavioral sickness symptoms (Reichenberg, 2001). The selective increase in peripheral cytokine levels in the absence of subjective central nervous system effects on cognitive or intellectual function suggested that changes in memory functions under these conditions would reflect objective endotoxin-induced alterations. Cortisol levels increased during the first and second testing periods, TNF-α and IL-6 peaked during the first testing period and decreased there-

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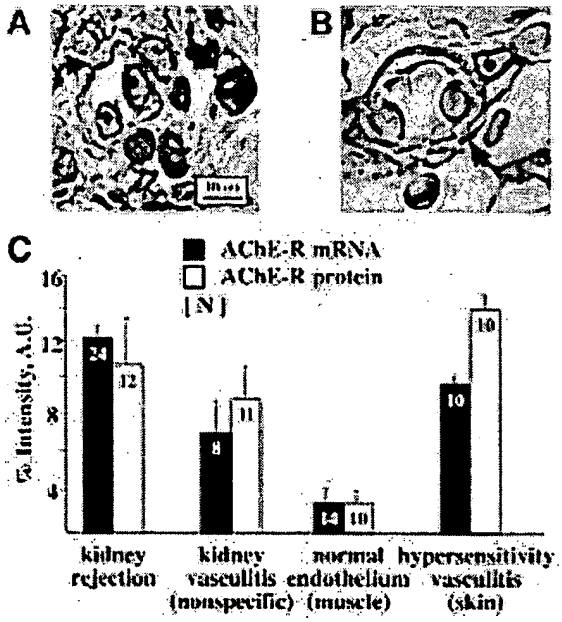

Fig. 3. AChE-R is expressed in human vascular endothelial cells from various tissues. (A) AChE-R mRNA. Shown are the results of *in situ* hybridization using a 5'-biotinylated cRNA probe selective for the AChE-R mRNA variant on sections of human vascular endothelial cells affected by an inflammatory process (skin hypersensitivity vasculitis; labeling is seen as pink color, red arrow). (B) AChE-R protein. Shown is an immunomicrograph of human kidney vascular endothelial cells from a patient with vasculitis, labeled with antibodies targeted at the AChE-R C-terminal peptide (red arrow). (C) Image analysis. Shown are average AChE-R mRNA and AChE-R protein labeling intensities (black and white columns, respectively), in kidney, skin, and muscle vascular endothelial cells (mean values ± S.E.M) as the percentage of red pixels, falling within a defined intensity range.

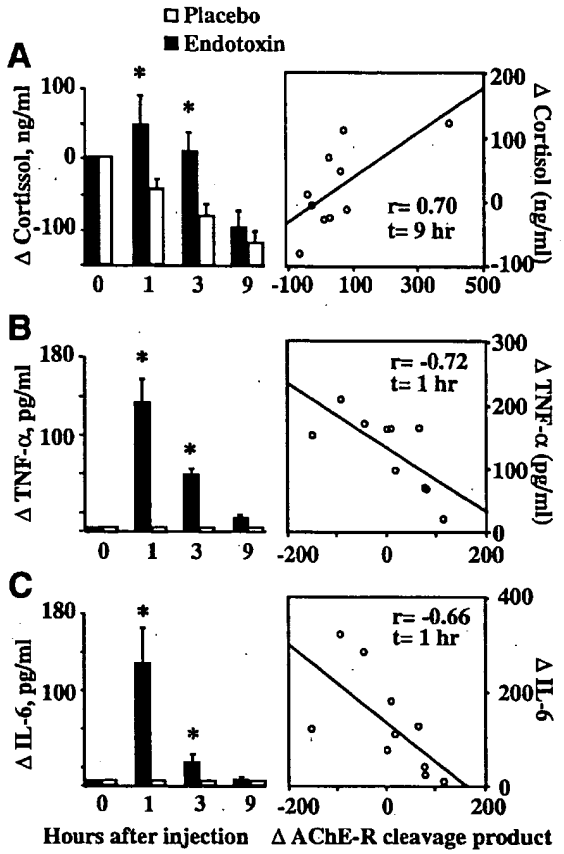

Fig. 4. Bidirectional associations between AChE-R cleavage and the changes in cortisol and cytokines. Shown are average ± S.E.M. changes with time (left) in the plasma levels of cortisol (A), TNF-α, (B) and IL-6 (C) of the 10 patients treated with endotoxin or placebo, and the associations (right) at the noted time points between these changes and the changes in AChE-R cleavage (measured by densitometric quantification of the C-terminus AChE-R cleavage product). r = correlation coefficient; t = time after injection.

after, and rectal temperature (not shown) peaked during the second period. These time-dependent effects were reflected by significant treatment-by-time interactions [$F(2,16) = 41.2, 10.6, 10.5, 3.2$, respectively, all $p < 0.05$, by H-F].

At each testing period, correlation analysis enabled the comparison between the biochemical and functional responses of tested individuals. Thus, endotoxin-induced AChE-R cleavage (computed as the change in a certain individual from the endotoxin to the placebo condition) was significantly ($p < 0.05$) and positively correlated with the secretion of cortisol, during the last testing period ($r = 0.70$) (Fig. 4A). AChE-R cleavage was significantly ($p < 0.01$) and negatively correlated with the secretion of TNF-α and IL-6 during the first ($r = -0.72$ and -0.66, respectively) (Fig. 4 B,C), but not later testing periods.

AChE-R Cleavage Is Associated with Endotoxin-Induced Impairments in Declarative Memory

Endotoxin administration decreased the performance in tests of declarative memory during all testing periods. This was reflected by decreased immediate recall of story items [$F(1,8) = 6.5$, $p <= 0.03$] (Fig. 5A) and reduced delayed story recall [$F(1,8) = 3.5$, $p <= 0.09$] (data not shown). Endotoxin-induced decrease in immediate and delayed recall

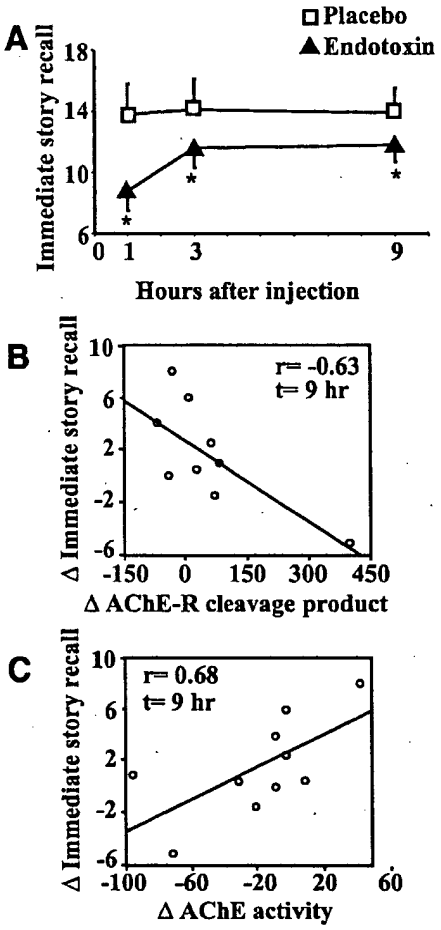

Fig. 5. Endotoxin impairs declarative memory. Shown are average ± S.E.M. values for the performance in the immediate story recall test of the endotoxin and placebo treated individuals at the noted time following treatment as well as the associations of the changes in these values at 9 h postinjection with the changes in AChE-R cleavage (B) and AChE activity (C).

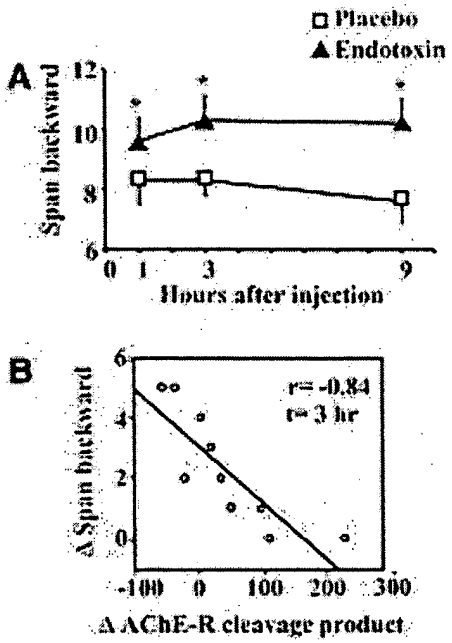

Fig. 6. Endotoxin-induced improvement in working memory. Shown are the performance values (average ± S.E.M.) in the span background test for the endotoxin and placebo treated individuals (A) and the association of the changes in this performance at 3 h postinjection with the changes in AChE-R cleavage (B). r = correlation coefficient, t = time after injection.

of story items was significantly ($p < 0.05$) and negatively associated with TNF-α and IL-6 secretion (r = –0.59 to –0.67) during the first, but not during other, testing periods (data not shown), suggesting the potential involvement of additional mechanism(s) in endotoxin-induced impairments in declarative memory. At the last testing period, the endotoxin-induced decrease in immediate recall of story items was significantly ($p < 0.05$) and negatively ($r = -0.63$) associated with AChE-R cleavage (Fig. 5B), indicating that the consequent increase in ACh levels, perhaps in conjunction with continuously suppressed cytokine production, interferes with declarative memory. This notion was supported by the positive ($r = 0.68$) association of declarative memory impairments with the decrease in AChE activity during the last testing period (Fig. 5C), when cytokine levels already receded, but not during earlier testing periods.

AChE-R Cleavage Association with Improved Working Memory

Endotoxin administration induced a significant improvement in working memory performance, reflected by an increased score in the digit span backward test during all testing periods [$F(1,8) = 12.3$, $p <= 0.008$] (Fig. 6A). No significant changes in the digit span forward test (assessing memory span) or on the attention test (Ruff 2 & 7 cancellation test) were evident (data not shown), emphasizing the selectivity of the observed differences.

The endotoxin-induced improvement in working memory performance showed no significant association with the secretion of TNF-α, IL-6 or cortisol, yet was negatively associated with AChE-R cleav-

age. Association was significant ($p < 0.05$) during the second and third testing periods ($r = -0.84$ and -0.64, respectively) (Fig. 6B and data not shown). Thus, subjects with a greater endotoxin-induced elevation in AChE-R cleavage (and, presumably, larger increases in ACh levels) showed both lower endotoxin-induced improvement in working memory functioning, and greater endotoxin-induced impairment in declarative memory.

Discussion

Administration of a low dose of endotoxin to healthy volunteers induces secretion of proinflammatory cytokines and cortisol, compromises cholinergic homeostasis, and alters memory. Both psychological (e.g., Maes, 1998), and physical (e.g., Goodman, 1990) stressors are likewise associated with the production of proinflammatory cytokines (including TNF-α and IL-6) in humans. Exposure to stressful stimuli exerts profound effects on cholinergic homeostasis in general and on the production and cellular distribution of AChE-R in particular. Therefore, experimental endotoxemia emerges as a valid model for studying the interactions between cytokines and the changes in cholinergic homeostasis (as those are reflected by AChE-R modulations) as well as the impact of these interactions on memory functions. No subjective feelings of illness were involved, so that the endotoxin-induced memory alterations could not be attributed to a perceived physical illness-associated distress. The selectivity of the observed memory changes was compatible with reports by others that cortisol does not affect attention, verbal executive function or vigilance (Lupien, 1999).

Scheme 1 presents the kinetic follow-up for the different parameters that were measured and the postulated associations between them, predicting potentially causal relationships between the induction of cytokines, hormone secretion, AChE modulations, and the resultant memory changes. Intriguing were the facts that, during the first testing period, the endotoxin-induced impairment in declarative memory was highest and correlated positively with cytokine secretion, whereas the improvement in working memory became prominent at 3 h posttreatment and showed no correlation with cytokine secretion. In contrast, both types of memory changes were significantly correlated with AChE-R cleavage, although cholinergic control over working memory seemed to begin earlier than for declarative memory (3 h vs 9 h postinjection, respectively).

At the first testing period, the endotoxin-induced cytokine secretion was at its peak, whereas the change in AChE-R cleavage was minimal. Nevertheless, the endotoxin-induced secretion of TNF-α and IL-6 was negatively associated with AChE-R cleavage during the first testing period, but not at later time points. This pattern is compatible with AChE-R cleavage and the resultant increase in ACh-suppressing cytokine secretion. Increased endotoxin-induced proteolysis (Fahmi and Chaby, 1994; Roberts and Jones, 1997) could explain both the enhanced cleavage of plasma AChE-R and the suppressed AChE activity. That ACh attenuates the production of proinflammatory cytokines (Tracey, 2001; Bernik, 2002) is compatible with causal involvement for circulation ACh in this process.

Unlike brain neurons and intestinal epithelium, which overproduce AChE-R under stressful insults (Shapira, 2000; Meshorer, 2002), vascular endothelial cells express AChE-R mRNA and its protein product under widely variable conditions. Therefore, vascular endothel is a potentially continuous source for plasma AChE-R. Within the plasma, soluble AChE-R can serve to control circulating ACh levels. After endotoxin administration, proteolytic cleavage of AChE-R notes the initiation of activity loss, perhaps reflecting enhanced degradation of the protein when it lacks its C-terminus. AChE-R cleavage apparently occurs close to the C-terminal site where the alternative AChE variants diverge from each other. Although the C-terminal domain is likely separated from the core structure that is essential for ACh hydrolysis (Soreq and Seidman, 2001), reduced hydrolysis should elevate ACh levels, suppressing inflammatory cytokines production (Borovikova, 2000).

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The appearance of a sharp band immunoreactive with antibodies targeted at the C-terminal peptide of AChE-R suggests the existence of an endopeptidase cleaving AChE-R at its C-terminus, and our mass spectrometry analysis points at the asparagine residue at position 564 as its tentative cleavage site. A mammalian cysteine peptidase, complementary to the plant asparaginyl endopeptidase legumain is known (Chen, 1997). This enzyme is a glycoprotein with strict specificity for hydrolysis of asparaginyl bonds. It possesses an inherent N-terminal signal peptide, N-glycosylation, and maximal activity at pH 5.8, suggesting that it functions in lysosomes and that its production by vascular endothel would increase under inflammation. It also includes an RGD sequence, indicating that it might interact with mem-

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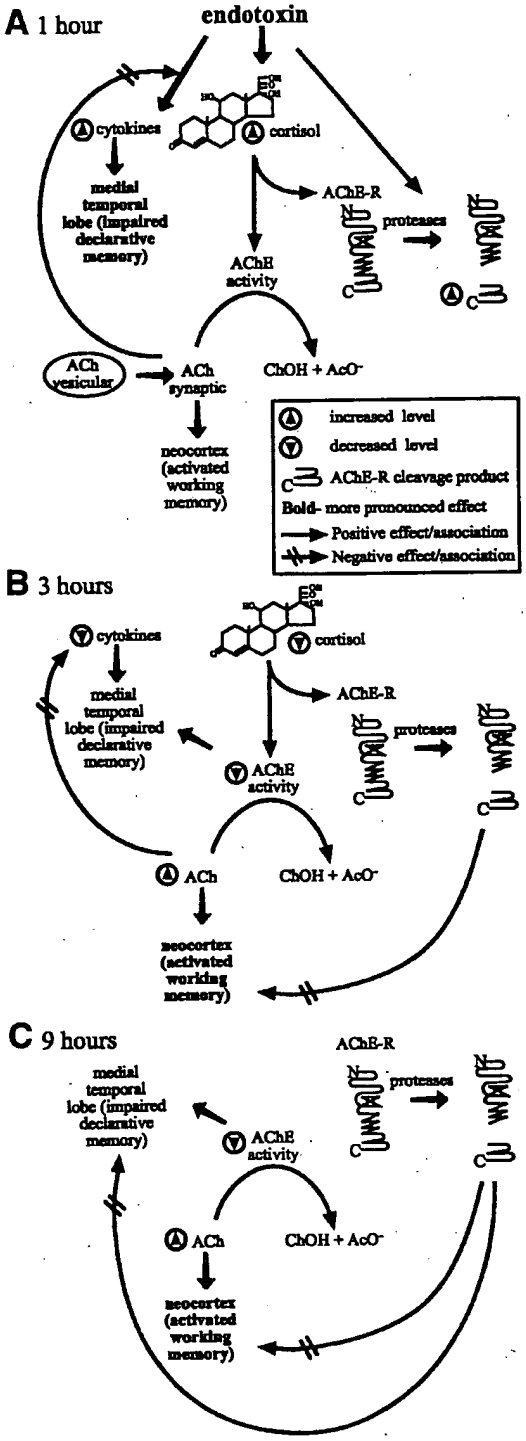
branes. The presence of legumain mRNA in human EST libraries of various tissue origins points to the possibility that it is causally involved in AChE-R cleavage.

Previous reports have documented decrements in declarative memory after endotoxin administration to healthy volunteers (Reichenberg, 2001), as well as after cytokine (especially interferon and IL-2) therapy (Meyers, 1999; Capuron, 2001), viral (e.g., influenza) infection (Capuron, 1999), or cortisol administration (de Quervain, 2000). In our study, the endotoxin-induced decrease in declarative memory performance was associated with cytokines secretion only in the first testing period. In contrast, it was associated with AChE activity and AChE-R cleavage levels during the last period, when cytokine concentrations have returned to baseline, yet the differences between AChE activity and AChE-R cleav-



Scheme 1. Endotoxin induces interrelated cytokine-cholinergic effects on memory. Shown are the cellular and biochemical events that were explored in our study and to which we attribute the observed changes in memory processes and the dynamic modifications in these changes during the posttreatment observation period. The thickness of arrows reflects the relative intensity of the relevant processes. **(A)** At 1 h posttreatment, endotoxin induces the release of cytokines, cortisol, and proteases. Cytokines elevation associates with impaired declarative memory, which is a medial temporal lobe-associated phenomenon. Cortisol induces AChE-R production, which elevates the immunopositive AChE-R amounts in plasma. Vesicular ACh is released into the synaptic cleft, where it affects neuronal electrophysiology and may improve working memory, which is a neocortex-associated property. In the periphery, ACh begins to suppress cytokines production in macrophages (circular arrow). **(B)** At 3 h posttreatment, proteases release a C-terminal fragment of 36 amino acids in length from AChE-R and initiate further destruction, followed by decreases in AChE activity. Endotoxin is already gone, and ACh effectively suppresses cytokines production; increased ACh levels (reflecting enhanced secretion and the decrease in AChE's hydrolytic activity) are probably associated with activated working memory, whereas the elevation in AChE-R cleavage product is associated with a lower working memory improvement. **(C)** At 9 h posttreatment, cortisol is gone as well. However, the persistent, although slow decrease in AChE activity is associated both with the impaired declarative memory and, probably through ACh increases, with the activated working memory. The steady increase in AChE-R cleavage product is now associated both with a greater impairment in declarative memory and with lower improvement in working memory.

age were maximal between the endotoxin and the placebo conditions. These findings may suggest that immune-mediated processes are prominent in the early endotoxin-induced memory impairments, whereas the later effects are probably mediated by the cholinergic system.

At first sight, the present finding that impaired memory is associated with higher change in and lower levels of plasma AChE activity appears contradictory to the putative role of AChE in the brain, as ample research indicates that AChE inhibitors attenuate the memory impairments in hypocholinergic animals (Ogura, 2000) and in Alzheimer's disease patients (Weinstock, 1995; Arendt, 2001; Palmer, 2002). However, there are at least two distinct AChE proteins in the mammalian brain, AChE-S and AChE-R (Soreq and Seidman, 2001). We have recently found that lower levels of synaptic AChE-S, in normal untreated FVB/N mice, as well as chronic overexpression of AChE-R in transgenic mice, were both associated with lower memory performance (Cohen, 2002). Reduced plasma AChE activity, as is the case under endotoxin exposure, possibly reflects a parallel reduction in the brain, which should indeed be associated with reduced declarative memory. Proper memory functioning would therefore depend on cumulative AChE-S and AChE-R activities, which should be neither deficient (as in the endotoxin-injected subjects) nor in excess (as in transgenic mice or under stress).

In contrast to the suppressed declarative memory, performance in the working memory test was improved under endotoxin as compared to placebo. Working memory, which involves temporary storage and manipulation of information necessary for cognitive functioning (Baddeley, 1992) and is facilitated in humans treated with cortisol (Stansbury, 2000) or with anticholinesterases (Palmer, 2002). This supposedly reflects facilitated signal-to-noise ratio in responding cortical single neurons (Furey, 2000), which increases the selectivity of perceptual responses, possibly by enlarging the response to afferent input and reducing background activity. It can be speculated that under conditions of acute stress, working memory is an important asset for survival. Thus, cholinergic-mediated improvement in working memory performance in the absence of attention deficits (as observed in the current study) might be adaptive. Animal studies show impaired working memory function in response to nicotinic antagonists (e.g., mecamylamine; Levin and Simon, 1998), and cholinergic antagonists, such as scopolamine, selectively interfere with encoding of new information, although not with its retrieval (Harder, 1998; Hasselmo, 1996). This provides support for causal relationship between working memory and the cholinergic system.

Correlation analysis enabled comparative evaluation of tested individuals with regard to the extent of change in their memory functions. Using this approach, the improvement in working memory performance was found to be negatively associated with AChE-R cleavage during the last two testing periods. This is compatible with the assumption that at these times AChE-R cleavage reflects the cholinergic status and that in individuals with particularly high increases in ACh levels, the endotoxin-induced improvement in working memory is limited. That cytokines and cortisol levels, as well as enzymatic AChE activity, appeared as not significantly associated with changes in working memory performance might be owing to the existence of other AChE variants (e.g., AChE-E) in the blood (Soreq and Seidman, 2001). Alternatively, or in addition, AChE-R cleavage may independently affect working memory performance after immune activation.

The endotoxin-induced changes in working and declarative memory support the suggested dissociation between brain structures associated with these functions (i.e., medial temporal lobe structures and declarative memory, neocortical areas and working memory (Helmstaedter, 1997; Squire, 1992). Declarative memory was impaired after lesions to the amygdalohippocampal complex but not the frontal cortex, whereas impaired working memory occurred following frontal, but not amygdalohippocampal lesions (Owen, 1996). Brain activation studies in humans further indicate frontal, but not amygdalohippocampal involvement, in working memory tasks (Cabeza and Nyberg, 2000). Thus, immune activation and cytokine secretion may affect these areas differentially, possibly modulating AChE-R cleavage (and, consequently, ACh levels) in a region-specific manner.

That cortisol levels were positively associated with AChE-R cleavage during the last testing period is consistent with both the glucocorticoid induction of AChE-R mRNA production (Meshorer, 2002), and the stress-induced AChE-R cleavage (Grisaru, 2001). Exposure of humans to glucocorticoids is indeed associated with decreased declarative memory performance (Kirschbaum, 1996; de Quervain, 2000), as

well as with improved working memory functioning (Furey, 2000; Stansbury, 2000). Enhancement of memory consolidation by glucocorticoids depends on coactivation of peripheral or central adrenergic mechanisms (Quirarte, 1997; Roozendaal, 1999) known to be activated by endotoxin (Linthorst, 1996; Zhang, 2000).

Our study demonstrates that changes in memory functioning after endotoxin exposure are coassociated with the induction of proinflammatory cytokines and AChE-R cleavage. The tentative pathway through which these changes may occur involves alterations in cholinergic neurotransmission and elevation in cytokine secretion (Scheme 1). These are associated with many medical conditions that involve inflammatory processes, particularly within the brain (e.g., stroke, brain trauma and neurodegenerative disease, such as vascular dementia) (McGeer and McGeer, 1995; Saito, 199; Levin and Simon, 1998). For example, closed head injury results in the production of TNF-α and other proinflammatory cytokines (Goodman, 1990; Trembovler, 1999) as well as in excessive accumulation of AChE-R within the brain (Shohami, 2000). Our findings suggest that cytokine-cholinergic interactions play an important role in the memory alterations that accompany these conditions, and may provide insights into the development of novel preventive and therapeutic procedures that will counteract the corresponding memory impairments without harming the improved capacities.

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References

American Psychiatric Association. (1994) *Diagnostic and Statistical Manual for Mental Disorders*, 4th ed, Washington, DC.

Arendt T. (2001) Alzheimer's disease as a disorder of mechanisms underlying structural brain self-organization. *Neuroscience*. **102**, 723–765.

Baddeley A. (1992) Working memory. *Science*. **255**, 556–229.

Beeri R., Andres C., Lev-Lehman E., Timberg R., Huberman T., Shani M., et al. (1995) Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. *Curr. Biol.* **5**, 1063–1071.

Beeri R., Le Novere N., Mervis R., Huberman T., Grauer E., Changeux J. P., et al. (1997) Enhanced hemicholinium binding and attenuated dendrite branching in cognitively impaired acetylcholinesterase-transgenic mice. *J. Neurochem.* **69**, 2441–2451.

Bernik T. R., Friedman S. G., Ochani M., DiRaimo R., Ulloa L., Yang H., et al. (2002) Pharmacological stimulation of the cholinergic antiinflammatory pathway. *J. Exp. Med.* **195**, 781–788.

Borovikova L. V., Ivanova S., Zhang M., Yang H., Botchkina G. I., Watkins L. R., et al. (2000) Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature*. **405**, 458–462.

Burrell R. (1994) Human responses to bacterial endotoxin. *Circ. Shock*. **43**, 137–153.

Cabeza R. and Nyberg L. (2000) Imaging cognition II: An empirical review of 275 PET and fMRI studies. *J. Cogn. Neurosci.* **12**, 1–47.

Capuron L., Lamarque D., Dantzer R., and Goodall G. (1999) Attentional and mnemonic deficits associated with infectious disease in humans. *Psychol. Med.* **29**, 291–7.

Capuron L., Ravaud A., and Dantzer R. (2001) Timing and specificity of the cognitive changes induced by interleukin-2 and interferon-alpha treatments in cancer patients. *Psychosom. Med.* **63**, 376–386.

Chen J. M., Dando P. M., Rawlings N. D., Brown M. A., Young N. E., Stevens R. A., et al. (1997) Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. *J. Biol. Chem.* **272**, 8090–8098.

Cohen O., Erb C., Ginzberg D., Pollak Y., Seidman S., Shoham S., Yirmiya R., et al. (2002) Neuronal overexpression of 'readthrough' acetylcholinesterase is associated with antisense-suppressible behavioral impairments. *Mol. Psychiatry* **7**, 874–885.

de Quervain D. J., Roozendaal B., Nitsch R. M., McGaugh J. L., and Hock C. (2000) Acute cortisone administration impairs retrieval of long-term declarative memory in humans. *Nat. Neurosci.* **3**, 313–314.

Erb C., Troost J., Kopf S., Schmitt U., Loffelholz K., Soreq H., and Klein J. (2001) Compensatory mechanisms enhance hippocampal acetylcholine release in transgenic mice expressing human acetylcholinesterase. *J. Neurochem.* **77**, 638–646.

Fahmi H. and Chaby R. (1994) Differential recovery of macrophages from endotoxin-tolerant states elicited by lipopolysaccharide and enzymatic treatments. *Immunol. Invest.* **23**, 243–258.

Furey M. L., Pietrini P., and Haxby J. V. (2000) Cholinergic enhancement and increased selectivity of perceptual processing during working memory. *Science*. **290**, 2315–2319.

Gibertini M. (1996) IL1 beta impairs relational but not procedural rodent learning in a water maze task. *Adv. Exp. Med. Biol.* **402**, 207–217.

Goodman J. C., Robertson C. S., Grossman R. G., and Narayan R. K. (1990) Elevation of tumor necrosis factor in head injury. *J. Neuroimmunol.* **30,** 213–217.

Green P. and Allen L. M. (1995) *Manual for the CogniSyst Story Recall Test.* CogniSyst Inc., Durham, NC.

Grisaru D., Deutsch V., Shapira M., Pick M., Sternfeld M., Melamed-Book N., et al. (2001) ARP, a peptide derived from the stress-associated acetylcholinesterase variant, has hematopoietic growth promoting activities. *Mol. Med.* **7,** 93–105.

Harder J. A., Baker H. F., and Ridley R. M. (1998) The role of the central cholinergic projections in cognition: implications of the effects of scopolamine on discrimination learning by monkeys. *Brain Res. Bull.* **45,** 319–326.

Hasselmo M. E., Wyble B. P., and Wallenstein G. V. (1996) Encoding and retrieval of episodic memories: role of cholinergic and GABAergic modulation in the hippocampus. *Hippocampus.* **6,** 693–708.

Hauss-Wegrzyniak B., Vraniak P. D., and Wenk G. L. (2000) LPS-induced neuroinflammatory effects do not recover with time. *Neuroreport.* **11,** 1759–1763.

Heeschen C., Weis M., Aicher A., Dimmeler S., and Cooke J. P. (2002) A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. *J. Clin. Invest.* **110,** 527–536.

Helmstaedter C., Grunwald T., Lehnertz K., Gleissner U., and Elger C. E. (1997) Differential involvement of left temporolateral and temporomesial structures in verbal declarative learning and memory: evidence from temporal lobe epilepsy. *Brain Cogn.* **35,** 110–131.

Kaufer D., Friedman A., Seidman S., and Soreq H. (1998) Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature.* **393,** 373–377.

Kim J. J. and Diamond D. M. (2002) The stressed hippocampus, synaptic plasticity and lost memories. *Nat. Rev. Neurosci.* **3,** 453–462.

Kirschbaum C., Wolf O. T., May M., Wippich W., and Hellhammer D. H. (1996) Stress- and treatment-induced elevations of cortisol levels associated with impaired declarative memory in healthy adults. *Life Sci.* **58,** 1475–1483.

Levin E. D. and Simon B. B. (1998) Nicotinic acetylcholine involvement in cognitive function in animals. *Psychopharmacology (Berl).* **138,** 217–230.

Linthorst A. C., Flachskamm C., Holsboer F., and Reul J. M. (1996) Activation of serotonergic and noradrenergic neurotransmission in the rat hippocampus after peripheral administration of bacterial endotoxin: involvement of the cyclo-oxygenase pathway. *Neuroscience* **72,** 989–997.

Lupien S. J., Nair N. P., Briere S., Maheu F., Tu M. T., Lemay M., McEwen B. S., and Meaney M. J. (1999) Increased cortisol levels and impaired cognition in human aging: implication for depression and dementia in later life. *Rev. Neurosci.* **10,** 117–139.

Maes M., Song C., Lin A., De Jongh R., Van Gastel A., Kenis G., et al. (1998) The effects of psychological stress on humans: increased production of pro-inflammatory cytokines and a Th1-like response in stress-induced anxiety. *Cytokine* **10,** 313–318.

Maier S. F. and Watkins L. R. (1998) Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychol. Rev.* **105,** 83–107.

McCaffrey R. J. and Lynch J. K. (1992) A methodological review of "method skeptic" reports. *Neuropsychol. Rev.* **3,** 235–248.

McGeer P. L. and McGeer E. G. (1995) The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res. Rev.* **21,** 195–218.

Meshorer E., Erb C., Gazit R., Pavlovsky L., Kaufer D., Friedman A., et al. (2002) Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. *Science* **295,** 508–512.

Meyers C. A. (1999) Mood and cognitive disorders in cancer patients receiving cytokine therapy. *Adv. Exp. Med. Biol.* **461,** 75–81.

Mullington J., Korth C., Hermann D. M., Orth A., Galanos C., Holsboer F., et al. (2000) Dose-dependent effects of endotoxin on human sleep. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278,** R947–R955.

Navia B. A., Jordan B. D., and Price R. W. (1986) The AIDS dementia complex: I. Clinical features. *Ann. Neurol.* **19,** 517–24.

Norusis M. J. (1994) *SPSS Advanced Statistics 6.1.* SPSS Inc., Chicago, IL.

Ogura H., Kosasa T., Kuriya Y., and Yamanishi Y. (2000) Donepezil, a centrally acting acetylcholinesterase inhibitor, alleviates learning deficits in hypocholinergic models in rats. *Methods Find Exp. Clin. Pharmacol.* **22,** 89–95.

Oitzl M. S., van Oers H., Schobitz B., and de Kloet E. R. (1993) Interleukin-1 beta, but not interleukin-6, impairs spatial navigation learning. *Brain Res.* **613,** 160–163.

Owen A. M., Morris R. G., Sahakian B. J., Polkey C. E., and Robbins T. W. (1996) Double dissociations of memory and executive functions in working memory tasks following frontal lobe excisions, temporal lobe excisions or amygdala-hippocampectomy in man. *Brain* **119,** 1597–1615.

Palmer A. M. (2002) Pharmacotherapy for Alzheimer's disease: progress and prospects. *Trends Pharmacol. Sci.* **23,** 426–433.

Perry C., Sklan E., Birikh K., Shapira M., Trejo L., Eldor A., et al. (2002) Complex regulation of acetylcholinesterase gene expression in human brain tumors. *Oncogene* **21,** 8428–8441.

Pollmacher T., Mullington J., Korth C., Schreiber W., Hermann D., Orth A., et al. (1996) Diurnal variations in the human host response to endotoxin. *J. Infect. Dis.* **174,** 1040–1045.

Pugh C. R., Kumagawa K., Fleshner M., Watkins L. R., Maier S. F., and Rudy J. W. (1998) Selective effects of peripheral lipopolysaccharide administration on con-

textual and auditory-cue fear conditioning. *Brain Behav. Immun.* **12,** 212–229.

Quirarte G. L., Roozendaal B., and McGaugh J. L. (1997) Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. *Proc. Natl. Acad. Sci. USA* **94,** 14048–14053.

Rachal Pugh C., Fleshner M., Watkins L. R., Maier S. F., and Rudy J. W. (2001) The immune system and memory consolidation: a role for the cytokine IL-1beta. *Neurosci. Biobehav. Rev.* **25,** 29–41.

Reichenberg A., Yirmiya R., Schuld A., Kraus T., Haack M., Morag A., et al. (2001) Cytokine-associated emotional and cognitive disturbances in humans. *Arch. Gen. Psychiatry.* **58,** 445–452.

Roberts J. C. and Jones S. B. (1997) Chromaffin cell epinephrine secretion mediated by a macrophage peptide: the role of endotoxin. *Shock* **7,** 211–216.

Roozendaal B., Nguyen B. T., Power A. E., and McGaugh J. L. (1999) Basolateral amygdala noradrenergic influence enables enhancement of memory consolidation induced by hippocampal glucocorticoid receptor activation. *Proc. Natl. Acad. Sci. USA* **96,** 11642–11647.

Ruff R. M. and Allen C. C. (1996) *Ruff 2 & 7 Selective Attention Test: Professional Manual.* Psychological Assessment Resources Inc., Lutz, FL.

Saito H., Togashi H., Yoshioka M., Nakamura N., Minami M., and Parvez H. (1995) Animal models of vascular dementia with emphasis on stroke-prone spontaneously hypertensive rats. *Clin. Exp. Pharmacol. Physiol. Suppl.* **22,** S257–S259.

Segal M. and Auerbach J. M. (1997) Muscarinic receptors involved in hippocampal plasticity. *Life Sci.* **60,** 1085–1091.

Shapira M., Tur-Kaspa I., Bosgraaf L., Livni N., Grant A. D., Grisaru D.,et al. (2000) A transcription-activating polymorphism in the ACHE promoter associated with acute sensitivity to anti-acetylcholinesterases. *Hum. Mol. Genet.* **9,** 1273–1281.

Shaw K. N., Commins S., and O'Mara S. M. (2001) Lipopolysaccharide causes deficits in spatial learning in the watermaze but not in BDNF expression in the rat dentate gyrus. *Behav. Brain Res.* **124,** 47–54.

Shohami E., Kaufer D., Chen Y., Seidman S., Cohen O., Ginzberg D., et al. (2000) Antisense prevention of neuronal damages following head injury in mice. *J. Mol. Med.* **78,** 228–236.

Soreq H. and Glick D. (2000) Novel roles for cholinesterases in stress and inhibitor responses, in *Cholinesterases and Cholinesterase Inhibitors: Basic, Preclinical and Clinical Aspects,* Giacobini E., ed., Martin Dunitz, London, pp. 47–61.

Soreq H. and Seidman S. (2001) Acetylcholinesterase—new roles for an old actor. *Nat. Rev. Neurosci.* **2,** 294–302.

Squire L. R. (1992) Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. *Psychol. Rev.* **99,** 195–231.

Stansbury K., Haley D., and Koeneker A. (2000) Higher cortisol values facilitate spatial memory in toddlers. Brief report. *Ann. NY Acad. Sci.* **911,** 456–458.

Sternfeld M., Shoham S., Klein O., Flores-Flores C., Evron T., Idelson G. H., et al. (2000) Excess "readthrough" acetylcholinesterase attenuates but the "synaptic" variant intensifies neurodeterioration correlates. *Proc. Natl. Acad. Sci. USA* **97,** 8647–8652.

Tabachnick B. G. and Fidell L. S. (2001) *Using Multivariate Statistics,* 4th ed, Allyn and Bacon, Boston, MA.

Thornton A. E., Raz N., and Tucke K. A. (2002) Memory in multiple sclerosis: contextual encoding deficits. *J. Int. Neuropsychol. Soc.* **8,** 395–409.

Tracey K. J., Czura C. J., and Ivanova S. (2001) Mind over immunity. *Faseb. J.* **15,** 1575–1576.

Trembovler V., Beit-Yannai E., Younis F., Gallily R., Horowitz M., and Shohami E. (1999) Antioxidants attenuate acute toxicity of tumor necrosis factor-alpha induced by brain injury in rat. *J. Interferon Cytokine Res.* **19,** 791–795.

Wechsler D. (1987) *Wechsler Memory Scale, Revised Manual,* The Psychological Corp., San Antonio, TX.

Weinstock M. (1995) The pharmacotherapy of Alzheimer's disease based on the cholinergic hypothesis: an update. *Neurodegeneration.* **4,** 349–356.

Willard L. B., Hauss-Wegrzyniak B., and Wenk G. L. (1999) Pathological and biochemical consequences of acute and chronic neuroinflammation within the basal forebrain cholinergic system of rats. *Neuroscience.* **88,** 193–200.

Winer B., Brown D., and Michels K. (1991) *Statistical Principles in Experimental Design,* 3rd ed, McGraw-Hill, New York.

Yirmiya R., Barak O., Avitsur R., Gallily R., and Weidenfeld J. (1997) Intracerebral administration of Mycoplasma fermentans produces sickness behavior: role of prostaglandins. *Brain Res.* **749,** 71–81.

Zhang Y. H., Lu J., Elmquist J. K., and Saper C. B. (2000) Lipopolysaccharide activates specific populations of hypothalamic and brainstem neurons that project to the spinal cord. *J. Neurosci.* **20,** 6578–6586.

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ORIGINAL RESEARCH ARTICLE

Stress-induced alternative splicing of acetylcholinesterase results in enhanced fear memory and long-term potentiation

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Stress insults intensify fear memory; however, the mechanism(s) facilitating this physiological response is still unclear. Here, we report the molecular, neurophysiological and behavioral findings attributing much of this effect to alternative splicing of the acetylcholinesterase (AChE) gene in hippocampal neurons. As a case study, we explored immobilization-stressed mice with intensified fear memory and enhanced long-term potentiation (LTP), in which alternative splicing was found to induce overproduction of neuronal 'readthrough' AChE-R (AChE-R). Selective downregulation of AChE-R mRNA and protein by antisense oligonucleotides abolished the stress-associated increase in AChE-R, the elevation of contextual fear and LTP in the hippocampal CA1 region. Reciprocally, we intrahippocampally injected a synthetic peptide representing the C-terminal sequence unique to AChE-R. The injected peptide, which has been earlier found to exhibit no enzymatic activity, was incorporated into cortical, hippocampal and basal nuclei neurons by endocytosis and retrograde transport and enhanced contextual fear. Compatible with this hypothesis, inherited AChE-R overexpression in transgenic mice resulted in perikaryal clusters enriched with PKCβII, accompanied by PKC-augmented LTP enhancement. Our findings demonstrate a primary role for stress-induced alternative splicing of the AChE gene to elevated contextual fear and synaptic plasticity, and attribute to the AChE-R splice variant a major role in this process.
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Introduction

Acute stress intensifies the evolutionarily advantageous memory of events that are potentially threatening to the organism.[1] A major challenge in neurocognition is to identify molecular mechanisms that underlie the enhanced formation of memory following stress exposure. The hippocampus is a critical component of the neuroanatomical stress circuit,[2] which is also involved in forming episodic, spatial and contextual memories.[3–7] Contextual fear conditioning, a procedure in which an animal learns to associate the neutral context of the training chamber with an aversive foot shock, involves the hippocampus, which participates in the storage of the memory representation of the context.[6,8–10] Stress-induced changes in hippocampal functioning require protein and RNA synthesis,[11] are associated with differential expression of immediate early genes (eg c-fos[12]) and involve alternative splicing of numerous transcripts, including potassium channels[13] and acetylcholinesterase (AChE).[14] Unlike the abundant AChE-S 'synaptic' variant, the stress-induced AChE-R possesses a hydrophilic C-terminus that is expected to be incapable of supporting membrane adherence. Neuronal AChE-R accumulation is accompanied by long-lasting hyperexcitation of glutamatergic activity[15] and prolonged conflict behavior.[16] To explore the possibility that changes in alternative splicing are critically involved in the stress-induced consolidation of fear memory, we tested the potential interrelationship between neuronal overproduction of the 'readthrough' AChE-R variant,[17] stress-enhanced fear memory and facilitated long-term potentiation

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(LTP) in the hippocampus.[18] Here, we report that, in the murine hippocampus, the stress-induced overproduction of AChE-R mRNA underlies enhanced contextual fear memory by facilitating synaptic plasticity in a process, which involves the interaction of PKCβII with the C-terminal peptide of AChE-R.

Materials and methods

Immunohistochemistry

Animals were deeply anesthetized and transcardially perfused at several time points (0, 1, 2, 3 or 24 h) after the end of the stress session. After the elimination of endogenous peroxidase activity and a preincubation step, 50 μm-thick coronal sections were incubated at 4°C with the rabbit anti-ARP antibody (1:600) for 48 h. Subsequently, sections were incubated with a biotinylated goat anti-rabbit antibody (1:200; Vector ABC kit) and with the ABC complex (Vector ABC kit). For visualization, DAB was used as a chromogen (Sigma fast tablet set). Sections were examined using light microscopy. The anteroposterior (AP) coordinates relative to bregma of the areas[19] included for detailed analysis were AP -1.34.[20]

Western blotting

Hippocampi were dissected out and immediately homogenized at 4°C with a plastic homogenizer, in a homogenization buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 4 mM EGTA, 15 mM sodium phosphate, 100 mM β-glycerophosphate, 10 mM sodium fluoride and a protease inhibitor cocktail tablet (Boehringer Mannheim, Germany). The insoluble material was removed by centrifugation at 13 000 *g* for 10 min at 4°C. Protein concentrations were determined by the Bradford method (BioRad, Munich, Germany). Equal amounts of protein for each group were separated on a 10% SDS gel and transferred to an Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA). The blot was probed using an anti-ARP antibody.[21] Western blots were developed using the chemiluminescence method.

Antisense oligonucleotides

mEN101 is a 20-mer oligodeoxynucleotide (5′-CTGCAATATTTTCTTGCA*C*C*C*-3′, stars denote 2′-oxymethyl groups) complementary to a sequence in exon E2 of mouse AChE mRNA.[15] InvEN101 is the inverted sequence (negative control oligodeoxynucleotide). A measure of 5 μM oligodeoxynucleotides was combined with 13 μM of the lipophilic transfection reagent DOTAP (Boehringer Mannheim, Germany) in an artificial cerebrospinal fluid (aCSF) and incubated for 15 min at 37°C prior to injection (of 25 ng in 1 μl).

Cannulation

Double guide cannulae (C235, Plastics One, Roanoke, VA, USA) were implanted into both lateral brain ventricles, using a stereotactic holder at Bregma AP 0 mm, lateral 1 mm and depth 3 mm. Alternatively, the cannulae were directed toward both dorsal hippocampi, AP -1.5 mm, lateral 1 mm and depth 2 mm.[19] Bilateral injections were performed using an infusion pump (CMA/100, CMA/Microdialysis, Solna, Sweden) at a constant rate of 0.33 μl/min. Cannula placement was verified *post hoc* in all mice by injection of methylene blue. For electrophysiological experiments, double-cannula placement was verified by unilateral methylene blue injection.[18]

Peptide

Mouse (m)ARP (GRRMEWGEQGMHKAARVGRR-GERWGAKHRV) was synthesized manually on 0.05 mmol of the 5-(4-*N*-Fmoc-aminomethyl-3.5-dimethoxyphenoxy)valeryl (PAL)-substituted polyethyleneglycol-polystyrene resin (Applied Biosystems), using *N*-α-Fmoc protected amino acids with the following side-chain protection: Arg(Pbf), Asn(Trt), Gln(Trt), Glu(OtBu), His(Trt) and Trp(Boc), obtained from Novabiochem. Couplings were carried out with four-fold excess of 9-fluorenylmethoxycarbonyl (Fmoc) amino acid, in the presence of 1 equivalent of 1,3-diisopropylcarbodiimide (DIC, Aldrich) and 2 equivalents of hydroxybenzotriazole (HOBt, Novabiochem) in freshly redistilled *N,N*-dimethylformamide (DMF). Coupling was monitored by bromophenol blue staining. The peptide was cleaved from the resin with 95/2.5/2.5 (v/v/v) trifluoroacetic acid/water/triisopropylsilane for 4 h, and products were purified by preparative reverse-phase HPLC on a Vydac C8 column, using a gradient of acetonitrile in 0.1% aqueous TFA. The purified product was characterized by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Voyager Mass Spectrometer using α-cyano-4-hydoxycinnamic acid matrix: mass calculated 3543.8, mass found 3544.2. PBAN[22] was from Phoenix Pharmaceuticals (Belmont, CA, USA).

Fluorescent peptides microinjection

All surgical treatments were performed under pentobarbital-sodium anesthesia. Hamilton syringe was directed toward the right lateral ventricle, using a stereotactic holder at Bregma AP 0.1 mm, lateral 1.2 mm, depth 2.2 mm. Injections were performed manually at a constant rate of ca. 0.33 μl/min. A time period of 5 h was allowed for endocytosis and peptide transport before the sacrifice period, followed by transcardial perfusion with 4% paraformaldehyde. Coronal brain sections (10 μm) were cut with a cryostat. The labeled peptides (NEN, Zaventhen, Belgium) were RITC-ARP (GRRMEWGEQGMHKAARVGRR-GERWGAKHRV) and FITC-ASP (DTLDEAERQWKA EFHRWSSYMVHWKNQFDHYSKQDRCSDL). Sections were inspected by means of confocal or fluorescent microscopy. Biotinylated ARP (NEN) was detected by rhodamine-labeled streptavidin (Sigma, St Louis, MO, USA), following 6 h incubation.

Drug treatment

mARP and PBAN were dissolved in aCSF solution and 0.25 μl of a 100 μM solution was injected per side.

Immobilization stress
An acute immobilization stress of mice consisted of taping their limbs to a plastic surface for 1 h.[23]

Fear conditioning
The single training trail consisted of placing the animal in a novel context (180 s), and administering a tone (30 s, 10 kHz, 75 dB SPL, pulsed 5 Hz) followed by a single footshock (US, 0.7 mA, 2 s, constant current). Under these conditions, the context served as the background stimulus. Background contextual fear conditioning but not foreground contextual fear conditioning, where the tone is omitted during training, has been shown to involve the hippocampus.[6] Freezing was recorded 24 h later in the same fear conditioning box for 180 s without tone presentation.[18]

Slice preparation and electrophysiology[18]
Hippocampal slices were obtained from 3–6 month-old male BALB/*c*, FVB/N or transgenic mice. Animals were killed by cervical dislocation. Brains were rapidly removed and placed in cold (2°C) artificial CSF (aCSF) consisting (in mM) of 124 NaCl, 5 KCl, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$ and 10 glucose (equilibrated with 95% O_2/5% CO_2). A bipolar, stimulating electrode placed in the stratum radiatum of the CA3 region of the slice (400 μm) was used to activate Schaffer collateral/commissural fiber synapses onto CA1 pyramidal cells. Extracellular field potentials were recorded with an aCSF-filled glass microelectrode placed in the stratum radiatum (electrode resistance up to 5 MΩ). Presynaptic fiber stimulation was set to evoke baseline fEPSPs ~50% of the maximal fEPSP amplitude. HFS-LTP was induced by three trains of 1 s, 50 Hz tetanic stimulation with 20 s intertrain interval, pulse width doubled to 0.1 ms in the tetanus. The TBS consisted of 5 × 100 Hz bursts (five diphasic pulses per burst) with a 200 ms interburst interval. All values are reported as mean ± SEM of all slices tested in the corresponding paradigm.

Fluorescence double labeling for confocal microscopy of PKCβII and ARP
The primary staining solution contained 0.3% Triton X-100, 0.05% Tween 20, 2% normal goat serum, 2% normal donkey serum, rabbit anti-ARP (1 : 100) and mouse-anti-PKCβII (Sigma, P8083), diluted 1 : 500. Secondary antibody solutions and preparation for microscopy were as detailed.[18] Slices were scanned by using a Bio-Rad MRC-1024 scanhead (Hemel Hempsted Herts.,UK) coupled to an inverted Zeiss Axiovert 135M microscope with a 40 × oil-immersion objective (NA 1.3). Excitation was at 488 nm (using 10% of a 100 mW laser). Fluorescence emission was measured by using a 580df32 bandpass interference filter (580 ± 16 nm) for detecting tetramethylrhodamine and a 525/40 filter for detecting fluorescein. The confocal iris was set to 3 mm. The conditions of scanning took into consideration the overlap of fluorescein fluorescence with the rhodamine filter (as was determined by control experiments). Images were then further processed with Image Pro Plus 4.01 program (version 4.0, Media Cybernetics, Silver Spring, MD, USA).

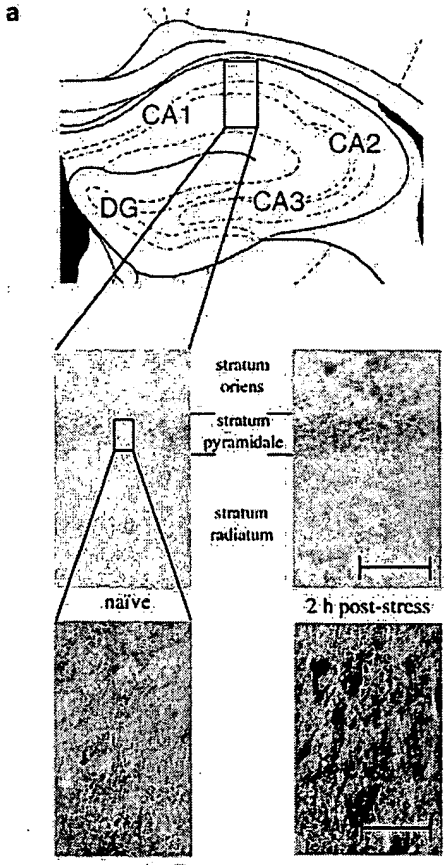
Statistics
Statistical comparisons were made by using unpaired Student's *t*-test and ANOVA. Significance was determined at the level of $P<0.05$ or 0.01.

Results

Immobilization stress induces transient alternative splicing of AChE in hippocampal neurons
Staining of hippocampal CA1 neurons from BALB/*c* mice with an anti-AChE-R antibody[21] revealed significantly increased labeling intensity of the stratum pyramidale and stratum radiatum 1, 2 and 3 h after 1 h immobilization, as compared to naïve mice (Figure 1a–c). This was compatible with the rapid post-stress increase of hippocampal AChE activities.[13] In the stratum pyramidale staining was maximal 2 h after immobilization and returned to baseline within 24 h (Figure 1b), demonstrating the transient nature of this response. In contrast, the labeling intensity of stratum radiatum was maximal at 24 h after immobilization (Figure 1c).

Enhanced memory of contextual fear depends on stress-induced AChE-R elevation
Following immobilization stress, RT–PCR analysis demonstrated a three-fold increase of AChE-R mRNA, using HPRT mRNA as control (Figure 2a). To study the implications of AChE-R mRNA upregulation for fear conditioning, we employed mEN101, an antisense oligonucleotide inducing murine AChE-R mRNA downregulation.[15] When intracerebroventricularly (i.c.v.) injected 15 min before immobilization, mEN101 selectively limited the stress-induced accumulation of AChE-R mRNA and protein to less than half of its full scale (Figures 2a and b and 3). In contrast, neither mEN101 nor the inversely oriented oligonucleotide invEN101 or the vehicle alone had any effect on AChE-S mRNA or the nonrelevant mRNA encoding the homologous protein butyrylcholinesterase (BuChE) (Figures 2a and b). Likewise, invEN101 or vehicle did not affect the level of the AChE-R mRNA or protein, as tested by RT-PCR and immunoblot analyses of hippocampal homogenates (Figures 2a and b and 3).

mEN101 diminishes, whereas the C-terminal peptide of mouse AChE-R intensifies, contextual fear
We have recently found that contextual fear conditioning was elevated if mice were trained 2 and 3 h after exposure to a stressful stimulus and assessed for memory 24 h later.[18] Our index of memory was the conditioned freezing response, absence of all movement except for respiration and heartbeat. Freezing was shown in mice that returned to the training



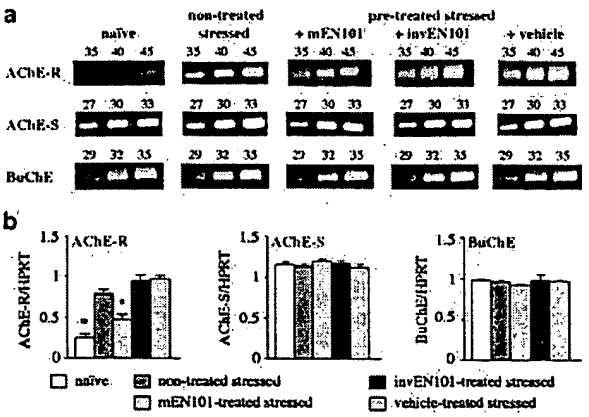


Figure 2 Antisense prevention of stress-induced AChE-R mRNA accumulation. RT-PCR analysis was performed on RNA extracted from the hippocampus of naïve or stressed mice, and from the hippocampus of mice that were injected 15 min before the stress session with the antisense agent (mEN101) that induces destruction of AChE-R mRNA, or with an inversely oriented sequence (invEN101) or vehicle. Each reaction mixture contained a set of primers specific for the cDNA of hypoxantine-phosphoribosyl-transferase (HPRT), an enzyme constitutively expressed at a low and constant level in the central nervous system, and widely used as internal control. (a) Bands reflect the levels of AChE-R mRNA, AChE-S mRNA and BuChE mRNA 2 h after the end of stress exposure. The numbers above each band indicate the cycle number. (b) Bar graphs show the ratio of mRNA band intensities calculated from densitometric analysis of a single cycle (AChE-R: 45, AChE-S: 33, BuChE: 35) verified to be within the linear range of product accumulation, divided by those of the coamplified HPRT product (mean ± SEM; 9–11 mRNA samples per group). Statistically significant differences: $^*P<0.01$ *vs* stressed animals.

context, in which they were previously exposed to a footshock.[24] Injection of mEN101 totally prevented the increased freezing response after stress, unlike invEN101 or vehicle injection that were ineffective (Figure 4).

A synthetic version of the C-terminal peptide unique to human AChE-R, hARP, has been shown to mimic the stress effect on proliferation of myeloid

Figure 1 Acute immobilization stress induces AChE-R upregulation in hippocampal CA1 neurons. (a) Top, schematic representation of the analyzed hippocampal brain area. Middle, AChE-R immunoreactivity in 50 μm coronal sections from the hippocampal CA1 area of naïve (left) and stressed (2 h after 1 h immobilization) mice (right; scale bar=100 μm) is shown. Bottom, higher magnification images of the framed regions (scale bar=25 μm). Bars represent densitometric analysis (mean ± SEM; both hippocampi of n=5 per group) of AChE-R-positive cells in the stratum pyramidale (b) and stratum radiatum (c), as seen in the middle segment under (a). Statistically significant differences: $^*P<0.05$ *vs* naïves.

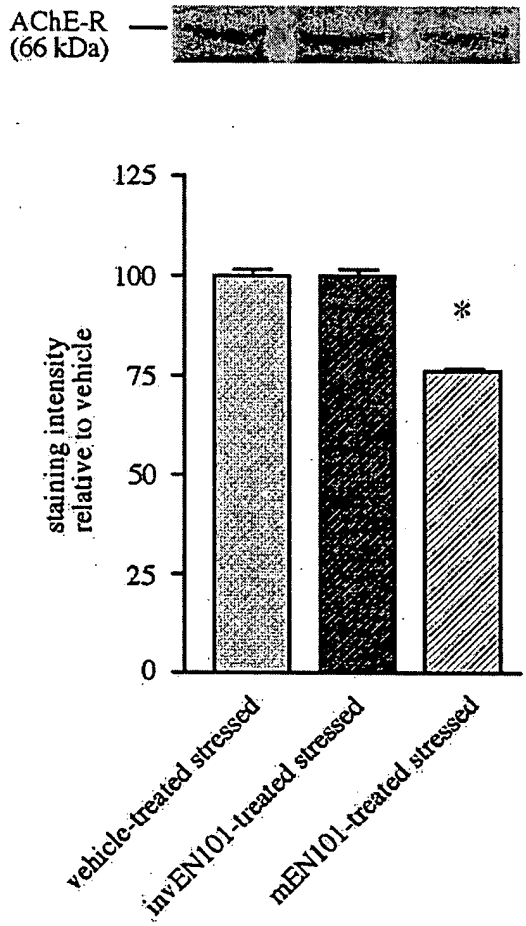


Figure 3 Downregulation of post-stress AChE-R protein levels by antisense treatment. A representative immunoblot reflecting AChE-R protein levels in hippocampi homogenates from stressed animals injected with mEN101, invEN101 or vehicle 15 min before stress exposure is shown. The hippocampi were removed and homogenized 2 h after the end of the stress session. Bars represent mean band intensities ± SEM for hippocampal homogenates (n=5, $^*P<0.001$).

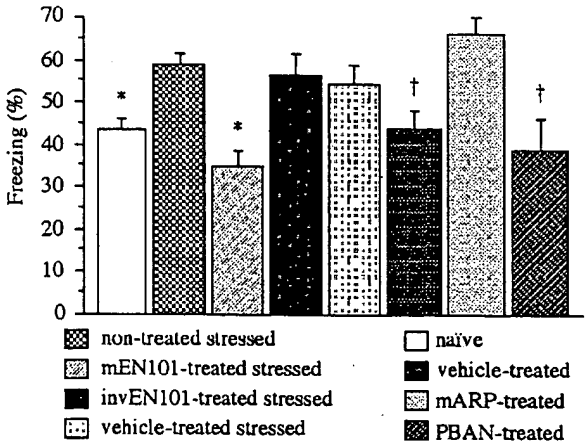


Figure 4 AChE-R elevation enhances contextual fear conditioning. Naïve (n=27), stressed (n=25) and mice injected i.c.v. 15 min before stress exposure with mEN101 (n=11), invEN101 (n=11) or vehicle (n=9) were trained 2 h after the end of the stress session or 2 h after intrahippocampal injection of vehicle (n=8), mARP (n=8) or PBAN (n=5) in the context-dependent fear-conditioning paradigm. Freezing was measured in the retention test performed 24 h after training. Statistically significant differences: $^*P<0.01$ *vs* stressed animals, $^\dagger P<0.01$ *vs* mARP-treated animals.

progenitor cells.[22] Therefore, we analysed whether elevated contextual fear observed under AChE-R overexpression could be mimicked by mARP, a synthetic peptide with the sequence of the mouse AChE-R C-terminus. FVB/N mice were first microinjected with rhodamine-labeled mARP to the lateral ventricle. Cortical and hippocampal neurons near the injection site displayed rhodamine fluorescence signals, likely reflecting ARP diffusion or local uptake (Figure 5a and b). However, neurons in areas distant from the injection site, such as the parietal cortex and the basal forebrain, were stained as well, suggesting endocytosis and retrograde transport of ARP (Figure 5c–e). An alternative labeling group did not affect the neuronal accumulation of ARP (Figure 5f). Moreover, a negative control peptide representing the C-terminus of murine AChE-S (mASP) displayed a conspicuously different labeling pattern. While mARP was localized in the cytoplasm, mASP accumulated in the neuronal nuclei (Figure 5g). Thus, injected mARP demonstrated a potential capacity to transduce signals to the neuronal cytoplasmic element(s). At the behavioral level, intrahippocampal (i.h.) mARP injection (2 nM) without conditioning had no effect on the motor activity of mice 24 h later (data not shown), but resulted in an elevated freezing response when mice were re-exposed to the conditioning context as compared to vehicle-treated controls. As a negative control peptide, we used the insect pheromone biosynthesis-activating neuropeptide (PBAN), with a molecular weight similar to mARP. PBAN, which had no effect on myelopoietic proliferation,[22] also showed no effect on contextual fear conditioning (Figure 4).

Elevated fear response involves enhanced hippocampal LTP

To test if AChE-R mRNA and protein upregulation after acute stress enhance conditioned fear by altering synaptic plasticity, we measured hippocampal LTP. In the present experiments, the Schaffer collateral pathway was stimulated to record theta-burst-induced LTP (TBS-LTP) from the CA1 stratum radiatum of hippocampal slices from stressed animals 2 h after 1 h immobilization. TBS-LTP was enhanced when

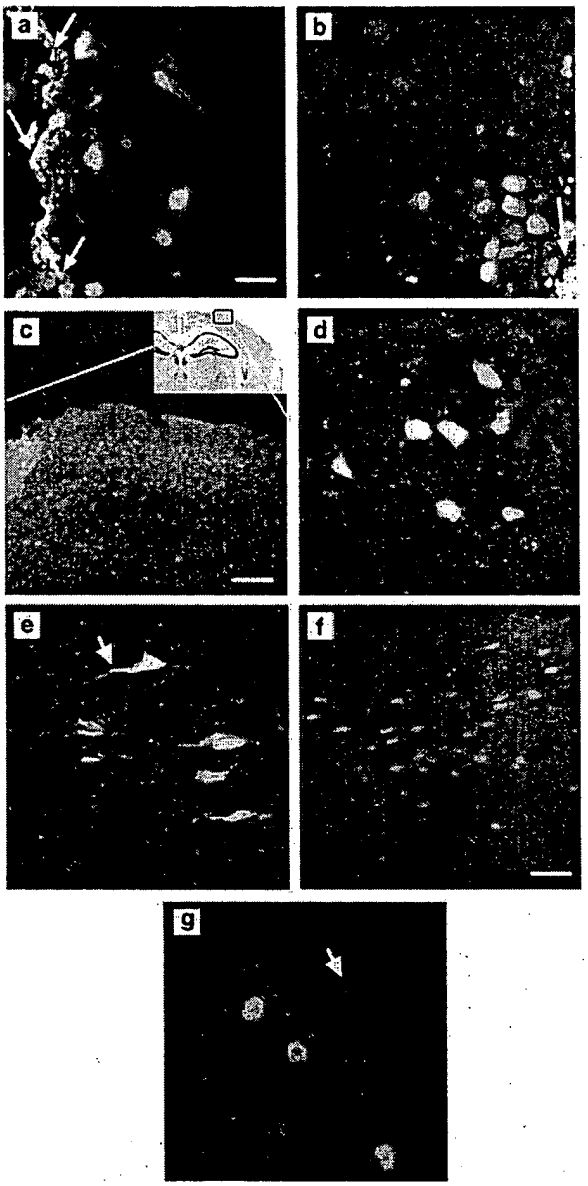


Figure 5 Neuronal endocytosis and retrograde transport of brain-microinjected synthetic mARP. Confocal section of cortex (a) and hippocampus (b) in which the injection needle is penetrated through (arrowheads). The nearby cells are stained for RITC-labeled ARP, probably via peptide diffusion. (c) Fluorescence microscopy of labeled areas distant from the injection site, for example, the parietal cortex. Confocal microscopy of stained cells from the basal forebrain; olfactory tubercle (d) and nucleus basalis of Meynert (e). Note the neural processes labeling with mARP (arrow). (f) Biotin and FITC-labeled mARP under simultaneous injection to the same animal (biotin is visualized with RITC-labeled streptavidin). In the nucleus basalis of Meynert, double-labeled cells appear yellow. (g) Simultaneous injection of RITC-labeled mARP and FITC-labeled mASP. While mARP is localized in the cytoplasm, mASP is localized in the nucleus. Note a cell labeled solely for RITC mARP (arrowhead). Scale bars: (a, b, d, e, g) 25 m; (c) 200 m; (f) 100 m.

compared to the response in brain slices from naïve animals. Facilitation of TBS-LTP was not detectable in slices from stressed mice pretreated with mEN101, whereas invEN101 was ineffective in blocking stress-mediated LTP facilitation (Figures 6a and b).

Persistent AChE-R overexpression induces a long-lasting upregulated LTP

Repeated forced swim episodes promote the dendritic translocation of hippocampal AChE-R mRNA in a long-lasting manner.[14] This finding raised the question if persistent AChE-R upregulation may contribute to enduring LTP facilitation and if such facilitation would persist in the maintenance phase, thought to be particularly relevant for long-term memory consolidation.[25] Therefore, we used high-frequency tetanic stimulation (HFS) to induce LTP in the stratum radiatum of naïve FVB/N transgenic mice overexpressing human AChE-R.[16,21] LTP was stably elevated by 40% even 100 min after LTP induction when compared to LTP of the parent strain mice (Figure 6c).

Enhanced PKC signal transduction in AChE-R-overexpressing mice

Neuronal AChE-R was recently found to tightly interact, through the scaffold protein RACK1, with PKCβII, the alternative splicing product of PKCβ, to increase its enzymatic activity and enlarge its density in hippocampal neurons.[16] In the hippocampus of AChE-R-overexpressing transgenic mice, immunohistochemical labeling of areas rich in PKCβII appeared as punctiform staining of higher cluster densities than in strain-matched FVB/N controls at the circumference of CA1 and dentate gyrus hippocampal neurons (Figures 7a and b). Confocal microscopy revealed in CA1 hippocampal neurons from these transgenic mice enlarged intracellular clusters composed of both AChE-R and PKCβII (Figure 7c). This finding was compatible with the assumption that the elevated LTP maintenance in transgenic mice may require interaction between the C-terminal domain of AChE-R and PKCβII.

We therefore investigated by activating PKC with phorbol dibutyrate (PDBu, 5 μM) whether the intensified staining of PKCβII correlated with enhanced PKC signaling in AChE-R transgenic mice. Indeed, 20 min administration of PDBu facilitated the synaptic field potentials more dramatically in hippocampal slices from AChE-R transgenic mice than from control FVB/N mice (Figure 7d). Further tetanic stimulation (20 min after PDBu wash) did not result in a significant additional potentiation, confirming that the synapses were fully potentiated by PKC activation in both WT and AChE-R Tg mice in accordance with previous studies (for example, Stanton[26]). The observed enhanced potentiation of synaptic transmission after phorbol ester treatment and tetanic stimulation in transgenic slices suggest that excess AChE-R leads to an increased strengthening of CA1 synapses due to higher PKC activity.

Discussion

This study provides evidence for a tight linkage between stress-induced alternative splicing in the hippocampus and the corresponding facilitation of fear conditioning. At the level of neuronal physiology, our study is compatible with the assumption that hippocampal LTP participates in the formation of contextual fear following a stressful experience.

Immunohistochemical staining of the stratum pyramidale indicated maximal expression of the stress-associated AChE splice variant AChE-R 2 h after immobilization. Mice conditioned at this time point revealed the highest levels of contextual fear during the retention test. This fear enhancement was prevented by antisense destruction of AChE-R mRNA, attributing a central role in stress-mediated contextual fear conditioning to AChE-R. Interestingly, the amount of AChE-R protein returned to baseline levels in the stratum pyramidale within 24 h, whereas it was maximal at that time point in the stratum radiatum. This observation was consistent with the reported stress-induced translocation of AChE-R mRNA from the nucleus of hippocampal CA1 neurons into dendrites.[14] In both, acutely stressed animals and animals persistently overexpressing AChE-R, LTP was intensified. However, HFS-LTP could only be elicited in slices from AChE-R transgenic mice, but not in slices from stressed mice, where it was inhibited.[18] This implies that additional components, other than AChE-R, participate in impeding HFS-LTP in slices from stressed mice. The involvement of additional, yet unidentified spliceosome proteins in fear memory consolidation is further indicated from the alternative pre-mRNA splicing at the ERK-MAP kinase pathway,[27] which is activated during fear conditioning.[28,29]

The physiological relevance of AChE-R overproduction may be multileveled, and should be discussed separately in the synaptic, intracellular and adaptive contexts. Following stress, AChE-R mRNA replaces AChE-S mRNA in neuronal processes in a manner associated with glutamatergic hyperexcitation.[14] AChE-R differs from the synaptic variant AChE-S in its C-terminal domain (ARP), which consists of 26 amino-acid residues as compared with 40 residues in AChE-S.[17] The unique ARP sequence, with no homologies in the database, is devoid of the cysteine residue that enables AChE-S to interact with the structural subunit PriMA,[30] and thus adhere to the synaptic membrane. Therefore, AChE-R would

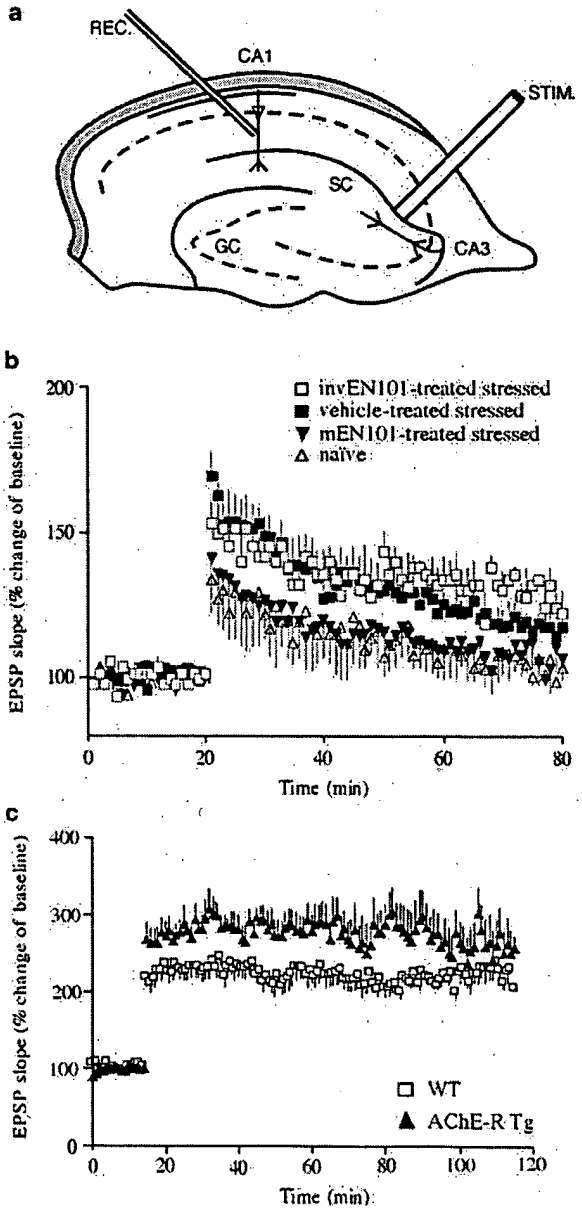


Figure 6 AChE-R facilitates LTP induction and maintenance in the mouse hippocampus. (a) Setup for hippocampal extracellular field recordings (REC) in the CA1 stratum radiatum upon stimulation (STIM) of the Schaffer collateral pathway (SC). (b) TBS-LTP elicited in slices from mice that were killed 2 h after 1 h immobilization (■) was significantly enhanced when compared to LTP induced in slices from naïve mice (△; seven slices, five mice; $P<0.001$). There was no statistical difference between stressed mice and mice that were preinjected with vehicle before stress exposure (six slices, five mice) (data not shown). The stress-induced enhancement was significantly attenuated when stressed mice were preinjected with mEN101 (▼; five slices, five mice; $P<0.001$). InvEN101 preinjection had no effect on stress-induced LTP facilitation (□; six slices, five mice). (c) Chronic effect of AChE-R overexpression. fEPSP changes from the baseline (%) of hippocampal slices from transgenic mice are shown (Tg; ▲; nine slices, six mice), reaching a value of 272±10% potentiation extent, significantly higher than the 228±8% potentiation extent in age- and strain-matched FVB/N control mice (□; 12 slices, nine mice, $P<0.05$).

primarily be expected to be secreted into the intercellular space, where it can rapidly hydrolyze the stress-elevated levels of acetylcholine.[13] However, the 534 residue core domain that is common to the two AChE variants shares sequence and function homologies with neuroligin, a postsynaptic cell adhesion molecule of excitatory synapses,[31,32] which triggers presynaptic development in contacting axons.[33] It is tempting to speculate that, under stress, excess AChE-R may compete with neuroligin, potentially impairing its interaction with β-neuroexin and modifying the subsequent activation of PSD95.[34]

Our finding that the synthetic peptide with the sequence of the mouse AChE-R C-terminus, mARP, mimicked the stress-associated effect of AChE-R demonstrates that the C-terminal domain, which is devoid of catalytic activity, is sufficient for promoting fear memories. The enhanced enzymatic AChE activity found shortly after stress[13] thus appears to be mainly responsible for clearing the elevated levels of acetylcholine released after acute stress.[35] In our current study, we indeed present endocytosis and active intracellular distribution of ARP. Thus, the involvement of AChE-R in eliciting fear memory emerges as a nonenzymatic activity, possibly involving intracellular protein–protein interactions. On the other side, it is conceivable that corticosterone mediates alternative splicing of the ACHE gene, not only in response to stress but also in response to learning itself. There is much evidence that corticosterone is involved in various learning paradigms including spatial orientation in the Morris water maze or contextual fear conditioning (for a review, see De Kloet[36]).

Since the C-terminus of AChE-R interacts intracellularly with the scaffold protein RACK1 and through it with PKCβII,[16] PKCβII might also contribute to enhanced fear conditioning after stress. In agreement, contextual fear conditioning of rats is associated with activation of hippocampal PKC and the translocation of PKCβII from the cytosol to the membrane,[37] and

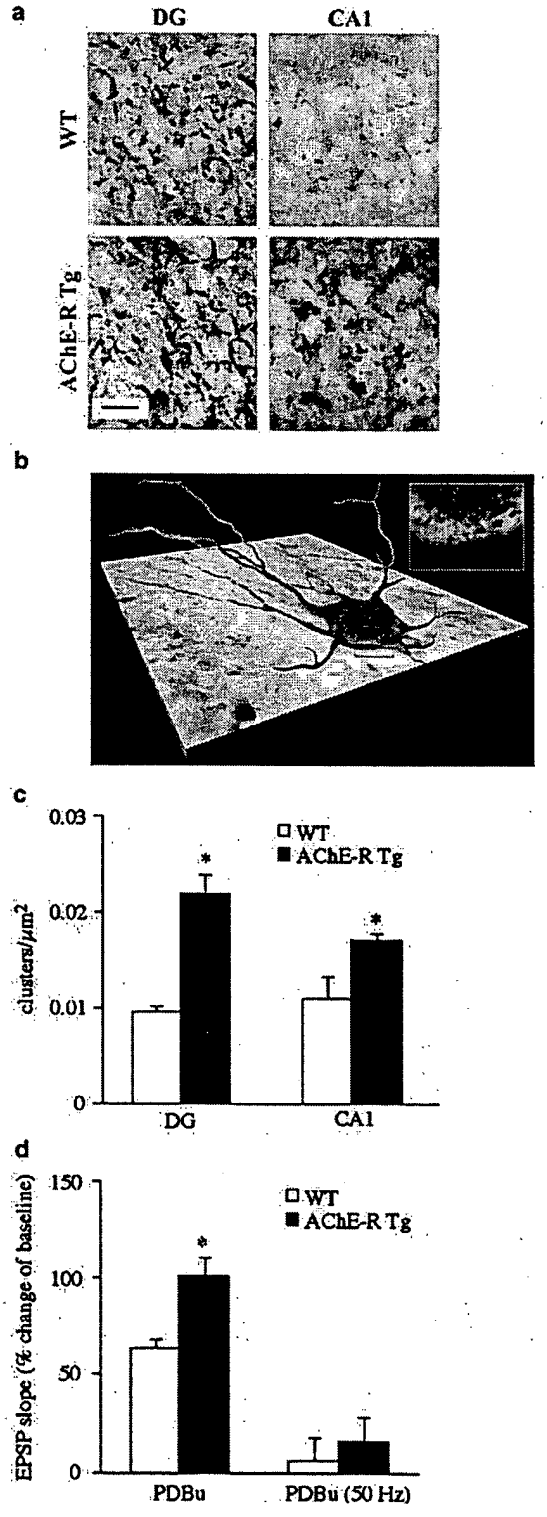


Figure 7 Activation of PKC induces LTP more profoundly under transgenic (Tg) AChE-R overexpression. (a) Facilitated PKC activation in the hippocampus of AChE-R transgenic mice. Typical pyramidal neurons in the hippocampal CA1 area and neurons in the granule cell layer of the dentate gyrus (DG) from WT and transgenic mice stained with a protein kinase C (PKC)βII antibody are shown. Scale bar, 25 μm. (b) Simulated immunomicrograph of a hippocampal neuron from a transgenic mouse overexpressing the stress-induced variant of acetylcholinesterase, AChE-R (green). The confocal images of clusters including AChE-R and PKCβII (red) are highlighted (inset). (c) Quantified labeling of the above sections. Sections from Tg (four sections, four mice) and WT (four sections, four mice) animals were quantified with significantly more clusters of PKCβII staining in transgenic neurons ($P<0.05$) in both DG and CA1 regions. Sections were 7 μm thick. (d) Summarized results of PDBu (5 μM) application for 20 min, which induced the facilitation of synaptic field potentials in AChE-R transgenic slices (nine slices, six mice), significantly higher than in WT (10 slices, six animals, $P<0.05$). Tetanic stimulation delivered after 20 min of PDBu wash did not induce further potentiation.

genomic disruption of mouse PKCβII causes inherited deficits in contextual fear responses.[38] However, PKCβII-deficient mice present apparently normal robust hippocampal LTP, albeit less sensitive to phorbol ester than that of control mice.[38] Thus, AChE-R-inducible PKCβII may serve as an important, but dispensable LTP modulator.

In summary, these data suggest that alternative splicing eliciting the accumulation of AChE-R and PKCβII is used by the hippocampus for mediating the effects of stress on fear conditioning and neuronal plasticity. It has been proposed that the neural mechanisms mediating adaptive fear are of clinical significance.[39–41] Thus, dysfunction of AChE-R or PKCβII or both are expected to be relevant to pathological conditions such as post-traumatic stress disorder (PTSD) and mood disorders.

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References

1 Kim JJ, Diamond DM. The stressed hippocampus, synaptic plasticity, lost memories. *Nat Rev Neurosci* 2002; **3**: 453–462.
2 McEwen BS. Corticosteroids and hippocampal plasticity. *Ann NY Acad Sci* 1994; **746**: 134–142.
3 Wilson MA, McNaughton BL. Dynamics of the hippocampal ensemble code for space. *Science* 1993; **261**: 1055–1058.
4 Rolls ET, Stringer SM, Trappenberg TP. A unified model of spatial and episodic memory. *Proc R Soc Lond B Biol Sci* 2002; **269**: 1087–1093.
5 Huerta PT, Sun LD, Wilson MA, Tonegawa S. Formation of temporal memory requires NMDA receptors within CA1 pyramidal neurons. *Neuron* 2000; **25**: 473–480.
6 Phillips RG, LeDoux JE. Lesions of the dorsal hippocampal formation interfere with background but not foreground contextual fear conditioning. *Learn. Mem.* 1994; **1**: 34–44.
7 Jensen O, Lisman JE. Position reconstruction from an ensemble of hippocampal place cells: contribution of theta phase coding. *J Neurophysiol* 2000; **83**: 2602–2609.
8 Moita MA, Rosis S, Zhou Y, LeDoux JE, Blair HT. Hippocampal place cells acquire location-specific responses to the conditioned stimulus during auditory fear conditioning. *Neuron* 2003; **37**: 485–497.
9 Barrientos RM, O'Reilly RC, Rudy JW. Memory for context is impaired by injecting anisomycin into dorsal hippocampus following context exploration. *Behav Brain Res* 2002; **134**: 299–306.
10 Sanders MJ, Wiltgen BJ, Fanselow MS. The place of the hippocampus in fear conditioning. *Eur J Pharmacol* 2003; **463**: 217–223.
11 Xu L, Holscher C, Anwyl R, Rowan MJ. Glucocorticoid receptor and protein/RNA synthesis-dependent mechanisms underlie the control of synaptic plasticity by stress. *Proc Natl Acad Sci USA* 1998; **95**: 3204–3208.
12 Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ. Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 1995; **64**: 477–505.
13 Kaufer D, Friedman A, Seidman S, Soreq H. Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature* 1998; **393**: 373–377.
14 Meshorer E *et al*. Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. *Science* 2002; **295**: 508–512.
15 Cohen O *et al*. Overexpression of 'readthrough' acetylcholinesterase is associated with antisense suppressible behavioral impairments. *Mol Psychiatry* 2002; **7**: 874–885.
16 Birikh K, Sklan E, Shoham S, Soreq H. Interaction of 'Readthrough' acetylcholinesterase with RACK1 PKCβII correlates with intensified fear induced conflict behavior. *Proc Natl Acad Sci USA* 2003; **100**: 283–288.
17 Soreq H, Seidman S. Acetylcholinesterase–new roles for an old actor. *Nat Rev Neurosci* 2001; **2**: 294–302.
18 Blank T, Nijholt I, Eckart K, Spiess J. Priming of long-term potentiation in mouse hippocampus by corticotropin-releasing factor acute stress: implications for hippocampus-dependent learning. *J Neurosci* 2002; **22**: 3788–3794.
19 Franklin KBJ, Paxinos G In: *The mouse brain in stereotaxic coordinates*. Academic Press: San Diego, 1997.
20 Nijholt I, Blank T, Ahi J, Spiess J. *In vivo* CREB phosphorylation mediated by dopamine and NMDA receptor activation in mouse hippocampus and caudate nucleus. *Brain Res Gene Expression Patterns* 2002; **1**: 101–106.
21 Sternfeld M *et al*. Excess 'read-through' acetylcholinesterase attenuates but the 'synaptic' variant intensifies neurodeterioration correlates. *Proc Natl Acad Sci USA* 2000; **97**: 8647–8652.
22 Grisaru D *et al*. ARP, a peptide derived from the stress-associated acetylcholinesterase variant has hematopoietic growth promoting activities. *Mol Med* 2001; **7**: 93–105.
23 Smith MA, Makino S, Kvetnansky R, Post RM. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci* 1995; **15**: 1961–1970.
24 Phillips R, LeDoux JE. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav Neurosci* 1992; **106**: 274–285.
25 Kandel ER. The molecular biology of memory storage: a dialogue between genes and synapses. *Science* 2001; **294**: 1030–1038.
26 Stanton PK. Transient protein kinase C activation primes long-term depression and suppresses long-term potentiation of synaptic transmission in hippocampus. *Proc Natl Acad Sci USA* 1995; **92**: 1724–1728.
27 Weg-Remers S, Ponta H, Herrlich P, Konig H. Regulation of alternative pre-mRNA splicing by the ERK MAP-kinase pathway. *The EMBO J* 2001; **20**: 4194–4203.
28 Schafe GE, Atkins CM, Swank MW, Bauer EP, Sweatt JD, LeDoux JE. Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *J Neurosci* 2000; **20**: 8177–8187.
29 Sananbenesi F, Fischer A, Schrick C, Spiess J, Radulovic J. Phosphorylation of hippocampal Erk-1/2, Elk-1, and p90-Rsk-1 during contextual fear conditioning: interactions between Erk-1/2 and Elk-1. *Mol Cell Neurosci* 2002; **21**: 463–476.
30 Perrier AI, Massoulie J, Krejci E. PRiMA: the membrane anchor of acetylcholinesterase in the brain. *Neuron* 2002; **33**: 275–285.
31 Song JY, Ichtchenko K, Sudhof TC, Brose N. Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc Natl Acad Sci USA* 1999; **96**: 1100–1105.
32 Grifman M, Galyam N, Seidman S, Soreq H. Functional redundancy of acetylcholinesterase and neuroligin in mammalian neuritogenesis. *Proc Natl Acad Sci USA* 1998; **95**: 13935–13940.
33 Scheiffele P, Fan J, Choih J, Fetter R, Serafini T. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 2000; **101**: 657–669.
34 Ichtchenko K, Hata Y, Nguyen T, Ullrich B, Missler M, Moomaw C, Sudhof TC. Neuroligin-1 a splice site-specific ligand for beta-neurexins. *Cell* 1995; **81**: 435–443.

35 Stillman MJ, Shukitt-Hale B, Coffey BP, Levy A, Lieberman HR. *In vivo* hippocampal acetylcholine release during exposure to acute stress. *Stress* 1997; **1**: 191–200.
36 De Kloet ER, Oitzl MS, Joels M. Stress and cognition: are corticosteroids good or bad guys? *Trends Neurosci* 1999; **22**: 422–426.
37 Young E, Cesena T, Meiri KF, Perrone-Bizzozero NI. Changes in protein kinase C (PKC) activity, isozyme translocation, and GAP-43 phosphorylation in the rat hippocampal formation after a single-trial contextual fear conditioning paradigm. *Hippocampus* 2002; **12**: 457–464.
38 Weeber EJ *et al*. A role for the beta isoform of protein kinase C in fear conditioning. *J Neurosci* 2000; **20**: 5906–5914.
39 Rosen JB, Schulkin J. From normal fear to pathological anxiety. *Psychol Rev* 1998; **105**: 325–350.
40 Gorman J, Kent J, Sullivan G, Coplan J. Neuroanatomical hypothesis of panic disorder, revised. *Am J Psych* 2000; **157**: 493–505.
41 Bouton ME, Mineka S, Barlow DH. A modern learning theory perspective on the etiology of panic disorder. *Psychol Rev* 2001; **108**: 4–32.

EXHIBIT I

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Differential Effects of Chronic Antidepressant Treatment on Swim Stress- and Fluoxetine-Induced Secretion of Corticosterone and Progesterone[1]

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ABSTRACT

Hypersecretion of cortisol occurs in numerous patients with major depression and normalizes with clinical recovery during the course of chronic antidepressant treatment. These clinical data suggest that investigation of the effects of antidepressant treatments on the regulation of the brain-pituitary-adrenal axis may assist in elucidating the therapeutic basis of antidepressant actions. In the present investigation, both swim stress and acute fluoxetine challenge increased release of corticosterone and progesterone to reflect an activation of the brain pituitary-adrenal axis. The effects of chronic antidepressant treatment (21 days) on corticosterone and progesterone secretion induced by these challenges were investigated. Chronic fluoxetine treatment (5 mg/kg/day) completely blocked the increased secretion of corticosterone and progesterone in response to the acute fluoxetine challenge. Chronic treatment with desipramine, imipramine or amytriptyline (15 mg/kg/day) also markedly attenuated fluoxetine-induced corticosterone and progesterone secretion. However, chronic treatment with the monoamine oxidase inhibitors, phenelzine (5 mg/kg) and tranylcypromine (5 mg/kg), did not affect this hormonal response to acute fluoxetine challenge. Plasma levels of fluoxetine after acute challenge were not significantly different for the various chronic antidepressant treatment conditions from the chronic saline controls; therefore, an increase in the metabolism of fluoxetine can not explain the antagonism of the fluoxetine-induced hormonal response after chronic antidepressant treatment. In contrast to the effects of selected antidepressants on acute fluoxetine-induced steroid release, chronic treatment with imipramine (20 mg/kg/day), fluoxetine (5 mg/kg/day) or phenelzine (5 mg/kg) did not significantly alter this swim stress-induced corticosterone or progesterone secretion. Because chronic fluoxetine and tricyclic antidepressant drugs blocked the acute action of fluoxetine to increase adrenal cortical secretion, but did not alter swim stress-induced secretion of these steroids, we propose that distinct neurochemical mechanisms control fluoxetine and swim stress-induced steroid release. We speculate that the substantial adaptive response to those chronic antidepressant treatments, which minimize the effect of acute fluoxetine challenge to increase in corticosterone and progesterone secretion, may be relevant to the therapeutic actions of these drugs.

Several measures of brain-pituitary-adrenal axis function are elevated in major depressive illness (Ettigi and Brown, 1977; Holsboer and Barden, 1996). For example, a substantial portion of depressed patients have chronically elevated cortisol (Gibbons, 1964; Sachar, 1975) and also exhibit a subsensitive negative feedback system, as reflected by attenuated inhibition of cortisol secretion in response to dexamethasone (Stokes *et al.*, 1975; Carroll *et al.*, 1981). Additionally, in depressed patients, cerebral spinal fluid levels of CRF are elevated (Banki *et al.*, 1987; Nemeroff *et al.*, 1984) and CRF mRNA in the paraventricular nucleus of depressed suicide victims is markedly higher than in control subjects (Raadsheer *et al.*, 1995). Depressed patients also exhibit a supersensitive adrenocorticotropin response to systemically administered CRF (Holsboer *et al.*, 1987; Schmider *et al.*, 1995). Because stress results in activation of the hypothalamic-pituitary-adrenal axis (Ganong and Forsham, 1960), the well-documented disturbances in the control of corticoid function in depression suggest that depressed patients may be in a state of chronic stress (Rubin *et al.*, 1987). In the course of chronic antidepressant drug treatment, normalization of adrenal cortical secretion precedes or coincides with clinical recovery from depression (Linkowski *et al.*, 1987; Steiger *et al.*, 1989; Souetre *et al.*, 1989).

In addition to stress, the brain-pituitary-adrenal axis is induced by acute treatment with serotonin uptake inhibitors and other drugs that increase the synaptic availability of serotonin, such as 5-hydroxytryptophan (Fuller and Snoddy, 1990). The magnitude of increased corticosterone secretion in

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ABBREVIATIONS: CRF, corticotrophin releasing factor; 5HT, serotonin; MAO, monoamine oxidase.

response to acute fluoxetine challenge is similar to that observed by various physiological stressors (Fuller and Snoddy, 1990). The acute corticosterone response to an antidepressant drug like fluoxetine seems paradoxical, because normalization of cortisol levels occurs with clinical recovery after chronic antidepressant therapy (Linkowski *et al.*, 1987; Steiger *et al.*, 1989; Souetre *et al.*, 1989).

The acute neuropharmacological properties of antidepressant drugs are well characterized. However, the immediate actions of these drugs on the function of the central nervous system can not account directly for their clinical efficacy, because chronic antidepressant administration is required for therapeutic effectiveness (Katz *et al.*, 1987; Klein and Davis, 1969). This requirement for chronic antidepressant administration for therapeutic effectiveness suggests that drug-induced neural adaptation is responsible for the therapeutic actions of this drug class (Heninger and Charney, 1987; Duman *et al.*, 1994). A common adaptive response produced by chronic treatment with several classes of antidepressant drugs, including tricyclics, MAO inhibitors and the selective serotonin uptake inhibitor fluoxetine, is a reduction in brain of levels of mRNA for CRF in animals (Brady *et al.*, 1991, 1992). Thus, neurochemical control of the brain-pituitary-adrenal axis presents a biological substrate to explore the mechanism by which antidepressant action affects the elevated activity of the brain pituitary-adrenal axis in depressive illness.

Although progesterone has been considered only as a female reproductive hormone, elevated levels of plasma progesterone accompany the increase in corticosterone after stress in male rats (Nequin *et al.*, 1975; Schaeffer and Aron, 1987; Deis *et al.*, 1989; Purdy *et al.*, 1991) and male humans (Breier and Buchanan, 1992). Stress-induced progesterone secretion in male and female rats is derived from the adrenal gland, because the response is abolished after adrenalectomy (Deis *et al.*, 1989; Nequin *et al.*, 1975; Purdy *et al.*, 1991). The physiological function of progesterone secreted from the adrenals in males and females is presently unclear. However, it is well established that systemically circulating progesterone can enter the brain and serve as a precursor for the synthesis of neurosteriods, which are potent allosteric moduators of γ-aminobutyric acid receptors (Majewska *et al.*, 1986; Morrow *et al.*, 1987; Purdy *et al.*, 1991; Paul and Purdy, 1992).

The purpose of the present investigation was to assess whether chronic antidepressant treatment would induce adaptive responses in the brain, such that corticosterone and progesterone responses to acute challenge with fluoxetine and swim stress would be attenuated. The swim stress protocol used was the forced swim test, a behavioral screen for antidepressant drug activity (Porsolt *et al.*, 1978).

Materials and Methods

Animals and treatments. Male Charles River Sprague-Dawley (Raleigh, NC) rats were housed three per cage, given continuous access to food (Purina rat chow) and water, and were on a 12-hr light-dark cycle with lights off at 1900 hr. For chronic treatments, rats were injected i.p. once a day for 21 days with drugs dissolved in 0.9% saline. Drugs were prepared at 4 mg/ml to minimize peritoneal irritation. At the termination of the experiments, when rats weighed 300 to 380 g, they were sacrificed by decapitation between 1300 and 1500 hr for collection of trunk blood.

Chronic antidepressant treatment and hormonal responses to acute fluoxetine challenge. Rats were injected daily for 3 weeks with imipramine-HCl (15 mg/kg), fluoxetine (5 mg/kg), desipramine-HCl (15 mg/kg), amytriptyline (15 mg/kg), phenelzine (5 mg/kg) or tranylcypromine (7 mg/kg) before the hormonal response to a challenge dose of fluoxetine was assessed. The 5 mg/kg challenge dose of fluoxetine was chosen because preliminary studies demonstrated that this dose induced robust secretion of corticosterone and progesterone. Furthermore, previous work by Fuller and Snoddy (1990) had shown that this dose was slightly submaximal. Trunk blood was collected and stored on ice until centrifugation to allow collection of the serum. Previous work showed that peak levels of corticosterone are obtained 40 min after i.p. injection of fluoxetine and that the levels of the hormone remain high and constant from 40 to 60 min after injection (Fuller and Snoddy, 1990). Therefore, for the acute fluoxetine challenge, rats were injected with fluoxetine-HCl (5 mg/kg), 20 to 24 hr after the final saline or drug injection in the 3-week drug treatment series, and sacrificed 40 min later.

Chronic antidepressant treatment and hormonal measurement in the forced swim test. Rats were injected daily i.p for 3 weeks with 0.9% saline, imipramine-HCl (20 mg/kg) or fluoxetine-HCl (5 mg/kg). The dose of 20 mg/kg imipramine considered the maximal dose of the drug for chronic administration and in previous work was demonstrated to antagonize swim stress-induced Fos (Duncan *et al.*, 1996). Desipramine was not examined in this work with the swim test because imipramine is metabolized to desipramine and substantial levels of this demethylated metabolite are present in brain after administration of imipramine. After the chronic saline or antidepressant treatment, rats were processed in the forced swim test (Porsolt *et al.*, 1978). For the conditioning swim on the first day of the test, the rats were placed in the swim tanks (40 × 19 cm Plexiglas cylinders) containing water (25°C) at a depth of 16 to 17 cm for 15 min. The final drug or saline injection was given after the conditioning swim. On the next day, for the test swim, the rats were placed in the tanks (25°C water) for 5 min and the duration of immobility measured. Rats were sacrificed 30, 60 or 120 min after the test swim, and trunk blood was collected and stored on ice until centrifugation.

Measurement of serum corticosterone and progesterone. Serum levels of corticosterone and progesterone were measured with radioimmunoassay kits (ICN Biomedicals, Inc. Costa Mesa, CA) according to the manufacturer's instruction, with ^{125}I-corticosterone and ^{125}I-progesterone. Duplicate samples for each rat were assayed for each hormone. The approximate detection limits for the corticosterone and progesterone were 10 and 0.1 ng/ml, respectively. There was less than 1% cross-reactivity between corticosterone and progesterone for the respective antisera in the kits.

Measurement of serum fluoxetine. Serum fluoxetine and norfluoxetine metabolite were determined by a high-performance liquid chromatographic method. Fluoxetine and norfluoxetine were extracted from 100-μl serum samples with hexane/*n*-butylamine (9:1) after the addition of internal standard (imipramine) and pH adjustment with ammonium hydroxide. The organic phase was evaporated to dryness under a gentle stream of nitrogen at room temperature. After reconstitution with 75 μl of methanol, the sample was transferred to injection vials. Fluoxetine, norfluoxetine and internal standard were separated on a 3-μm (4.6 × 250 mm) Hypersil silica RP column (Keystone Scientific, Inc, Bellefonte, PA) and a mobile phase of acetonitrile/methanol/ammonium hydroxide (860:140:5) at 2.0 ml/min. Quantitation of each compound was performed with UV detection at 254 nm (Linear Instruments, Reno, NV) and Chrom Perfect chromatographic software (version 2.05, from Justice Innovations, Inc., Mountainview, CA). Standard curves were linear from 25 ng/ml, the lower limit of quantitation, up to 1000 ng/ml for both compounds. The within- and between-day coefficients of variation were <9%.

Statistics. Data were analyzed by two-way analysis of variance and *post hoc* comparisons were made with Tukey's test.

Results

Effects of acute fluoxetine challenge on secretion of corticosterone and progesterone. In accord with previous results (Stark *et al.*, 1985; Fuller and Snoddy 1990), administration of fluoxetine (5 mg/kg) induced a robust secretion of corticosterone (fig. 1) that was similar in magnitude to that induced by swim (compare with figs. 8–10). Additionally, just as with stress in male rats (Purdy *et al.*, 1991), acute fluoxetine administration resulted in an increase in progesterone (fig. 1). To determine whether the increased secretion of these steroids involved activation of the brain-pituitary-adrenal axis, the synthetic glucocorticoid dexamethasone was administered 4 hr before fluoxetine injection. Dexamethasone is a potent synthetic glucocorticoid that suppresses adrenal cortical secretion by activating negative feedback mechanisms in the brain and pituitary gland (Meikle and Tyler, 1977). Pretreatment of rats with dexamethasone completely blocked fluoxetine-induced increases in corticosterone and progesterone secretion (fig. 1). These data indicate that fluoxetine induces the secretion of these steroids *via* an action on the brain and/or the pituitary gland.

Effects of chronic and subchronic treatment with fluoxetine on corticosterone and progesterone secretion in response to acute fluoxetine challenge. To evaluate the effect of chronic fluoxetine on the fluoxetine-induced increase in steroids, rats were challenged with 5 mg/kg fluoxetine after chronic administration of 0.9% saline or fluoxetine (5 mg/kg/day for 21 days). The fluoxetine challenge dose was given 20 to 24 hr after the final injection in the chronic fluoxetine treatment series. Chronic treatment with fluoxetine completely blocked the effects of the acute fluoxetine challenge on both corticosterone and progesterone secretion (fig. 2). In contrast to the effects of chronic fluoxetine treatment, rats injected with fluoxetine for 1 day, and challenged with 5 mg/kg fluoxetine the next day, did not exhibit diminished hormonal responses to the fluoxetine challenge (fig. 3).

Effects of chronic treatment with other antidepressant drugs on acute fluoxetine-induced corticosterone and progesterone secretion. To determine whether chronic treatment with antidepressants having pharmacological properties different from fluoxetine would antagonize the corticosterone and progesterone response to acute fluox-

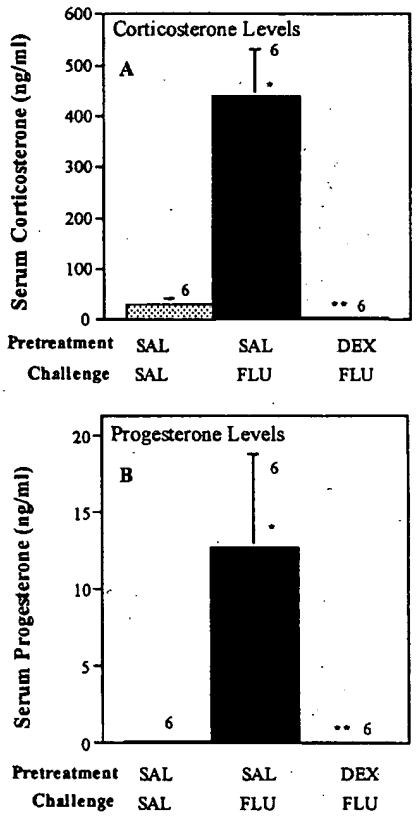

Fig. 1. Acute fluoxetine-induced increase in corticosterone and progesterone secretion: Effect of dexamethasone. Rats were pretreated with saline (SAL) or dexamethasone (DEX, 0.5 mg/kg) 4 hr before challenge with saline or fluoxetine (FLU). Rats were sacrificed 40 min after the saline or fluoxetine challenge. Data are means ± S.E.M. with $n = 6$ for each group. *P < .01 compared with SAL; **P < .01 compared with SAL-FLU.

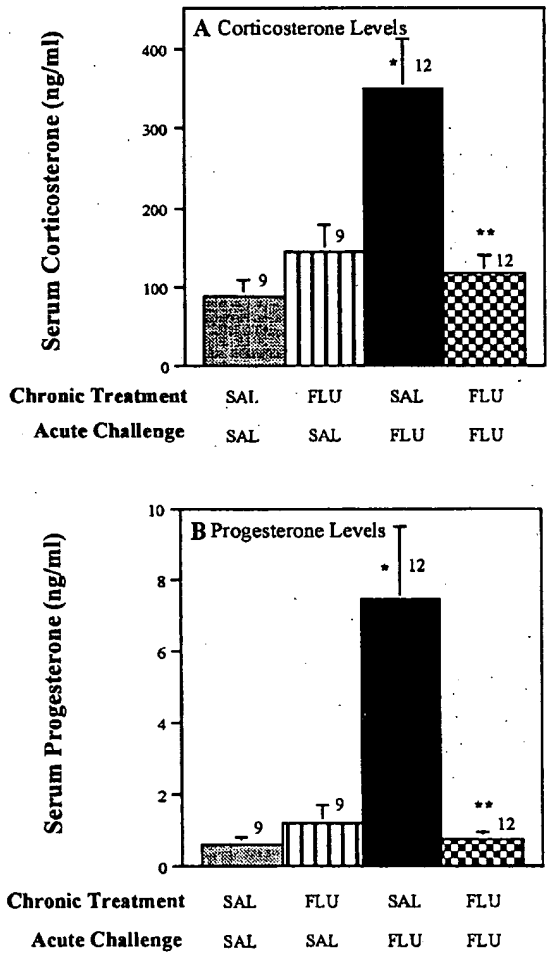

Fig. 2. Effect of chronic fluoxetine treatment on corticosterone and progesterone secretion in response to acute fluoxetine challenge. Rats were injected daily for 21 days with 0.9% saline or fluoxetine (5 mg/kg). Twenty-four hours after the final injection, a challenge dose of fluoxetine (5 mg/kg) or saline was administered and rats were sacrificed by decapitation 40 min later. Abbreviations: SAL, saline; FLU, fluoxetine. The number of rats in each group are given above the bars. Data are presented as the mean ± S.E.M. for each group. *P < .01 compared with SAL-SAL; ** P < .01 compared with SAL-FLU.

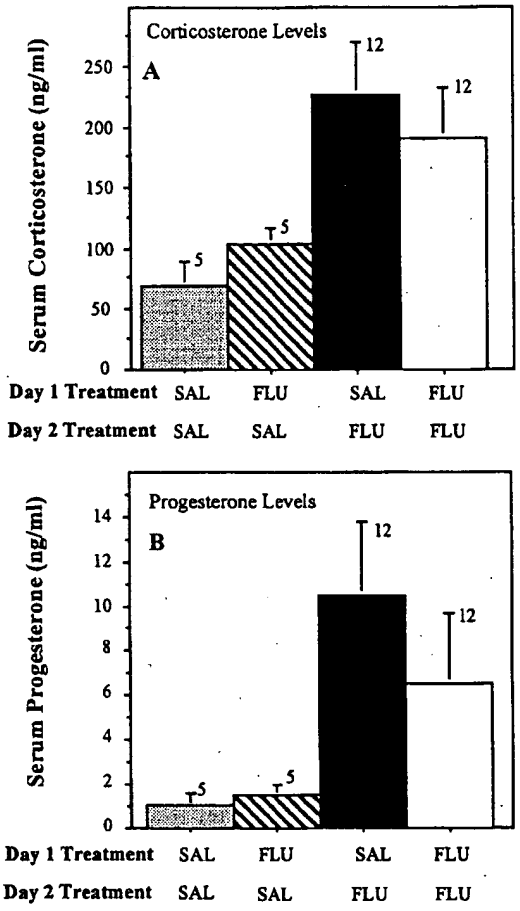

Fig. 3. Lack of effect of 1-day fluoxetine treatment on corticosterone and progesterone secretion in response to fluoxetine challenge. Rats were treated with fluoxetine (FLU, 5 mg/kg) or saline (SAL) on day 1 and challenged with saline or fluoxetine (5 mg/kg) on day 2, as indicated. SAL-FLU levels of corticosterone and progesterone after SAL-FLU were not significantly different from levels in the FLU-FLU-treated rats.

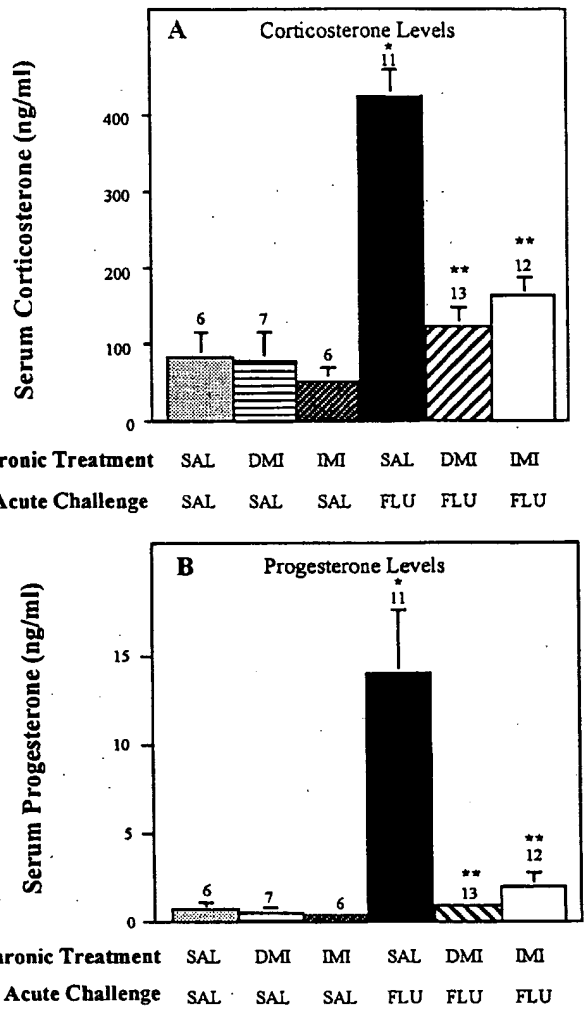

Fig. 4. Effect of chronic imipramine and desipramine treatment on fluoxetine-induced secretion of corticosterone and progesterone. Rats were injected daily for 21 days with 0.9% saline, imipramine or desipramine (15 mg/kg for each). Twenty-four hours after the final injection of desipramine and imipramine, a challenge dose of fluoxetine (5 mg/kg) was given and rats were sacrificed by decapitation 40 min later. The number of rats in each group are given above the bars. Data are presented as the mean ± S.E.M. for each group. Abbreviations: SAL, saline; IMI, imipramine; DMI, imipramine. *P < .01 compared with SAL-SAL; **P < .01 compared with SAL-FLU.

etine challenge, rats were treated chronically with various other clinically used antidepressants before an acute fluoxetine challenge. Antidepressants chosen for study were imipramine, desipramine, amytriptyline, tranylcypromine and phenelzine. Imipramine and amytriptyline inhibit the uptake of both serotonin and norepinephrine (Wood *et al.*, 1986), whereas desipramine has predominant actions on norepinephrine uptake with little effect on serotonin uptake (Randrup and Braestrup, 1977). Desipramine also was chosen for study to determine whether an antidepressant drug, which does not directly affect serotonin function, would induce an adaptive response in serotonergic control of the brain-pituitary-adrenal axis, as assessed by the acute fluoxetine challenge. Chronic administration of imipramine, desipramine (fig. 4) or amytriptyline (fig. 5) markedly attenuated corticosterone and progesterone secretion induced by acute fluoxetine challenge. In contrast to the blockade by the tricyclic antidepressants on fluoxetine-induced steroid secretion, chronic treatment with the MAO inhibitors, tranylcypromine and phenelzine, did not affect the hormonal response to acute fluoxetine challenge (fig. 5).

Effect of chronic antidepressant treatments on serum levels of fluoxetine. Because the reduced corticosterone and progesterone responses to acute fluoxetine challenge could have been caused by an altered metabolism of fluoxetine during the course of the chronic treatment of the antidepressants, plasma levels of fluoxetine were measured after chronic antidepressant treatments that antagonized the steroid response to fluoxetine. Serum fluoxetine levels after acute challenge were not significantly different in rats treated chronically with saline, fluoxetine, imipramine or desipramine (fig. 6). Serum norfluoxetine levels were significantly lower in rats treated chronically with desipramine and imipramine, but not fluoxetine (fig. 6). However, because fluoxetine levels were not reduced in the rats treated chronically with imipramine or desipramine, altered fluoxetine metabolism can not explain the attenuated hormonal responses to acute fluoxetine observed after chronic administration of these tricyclic antidepressants.

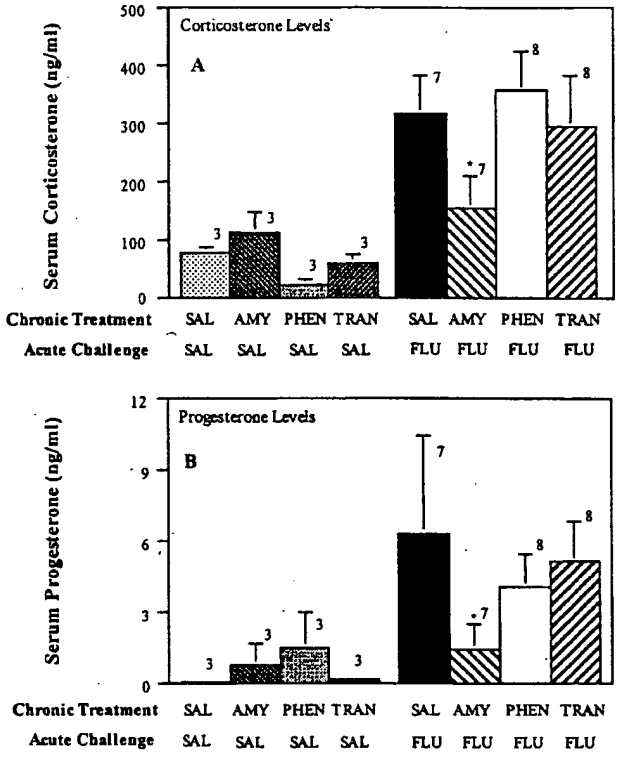

Fig. 5. Effect of chronic amytriptyline (AMY), phenelzine (PHEN) and tranylcypromine (TRAN) treatment on fluoxetine-induced secretion of corticosterone and progesterone. Rats were injected daily for 21 days with either 0.9% saline, AMY (15 mg/kg), PHEN (5 mg/kg) or TRAN (5 mg/kg). Twenty-four hours after the final injection of these chronic exposures, a challenge dose of fluoxetine (FLU; 5 mg/kg) was given and rats were sacrificed by decapitation 40 min later. The number of rats in each group are given above the bars. Data are presented as the mean ± S.E.M. for each group. **P < .05 compared to SAL-FLU.

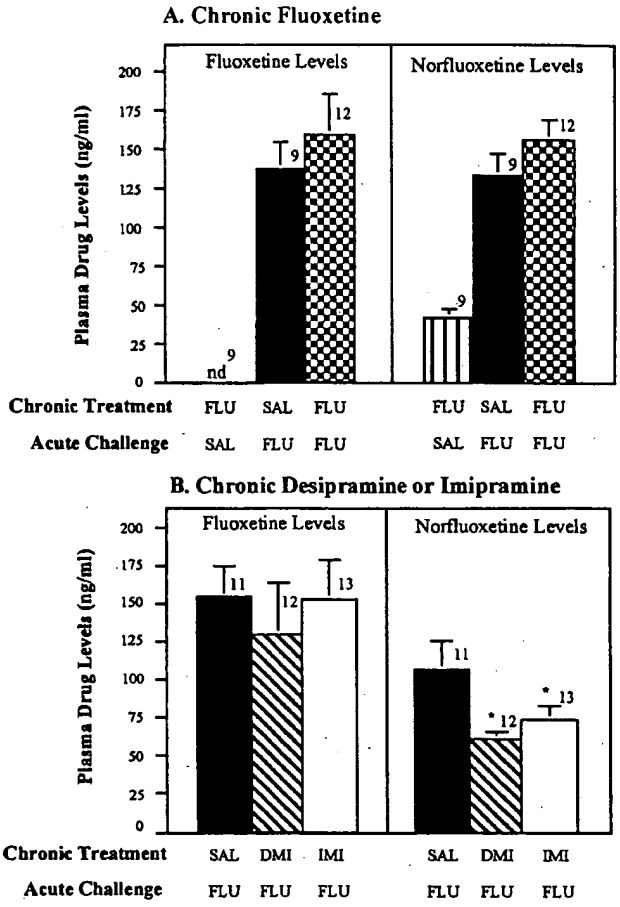

Fig. 6. Lack of effect of chronic treatment with desipramine (DMI), imipramine (IMP) or fluoxetine (FLU) on serum levels of fluoxetine after acute challenge. Rats were treated as described in figures 2 and 4. Fluoxetine and norfluoxetine levels were measured by HPLC with UV detection in serum obtained 40 min after acute challenge with fluoxetine. The number of rats in each group are given above the bars. Data are presented as the mean ± S.E.M. for each group. The norfluoxetine levels were reduced significantly in the chronic DMI- and IMP-treated rats, but not in the FLU-treated rats. *P < .05 compared with SAL-FLU.

Effects of chronic treatment with fluoxetine, imipramine and phenelzine on swim stress-induced immobility and corticosterone and progesterone secretion. To determine whether the marked reduction in fluoxetine-induced corticosterone and progesterone secretion after chronic antidepressant treatment was caused by generalized desensitization of the brain-pituitary-adrenal axis, the effects of chronic treatment with imipramine and fluoxetine on swim stress-induced secretion of these hormones was examined. No significant difference in basal or swim-induced corticosterone or progesterone levels was observed between rats treated chronically (3 weeks) with imipramine (fig. 7), fluoxetine (fig. 8) or phenelzine (fig. 9) and those treated with saline.

Even though no effect of chronic antidepressant treatment was found on swim-induced steroid secretion, all three antidepressant drugs reduce the duration of immobility in the swim test (fig. 10; Porsolt *et al.*, 1978). As reported previously (Detke *et al.*, 1995), behavioral responses to fluoxetine were qualitatively distinct from the behavioral response obtained with imipramine and phenelzine. After treatment with imipramine and phenelzine, rats jumped from the bottom of the swim tanks and attempted to vigorously climb the walls of the tanks for much of the nonimmobile time. In contrast, such behavior was not exhibited by the fluoxetine-treated rats, which exhibited less vigorous "escape-directed" behavior, but instead would swim around the tank without jumping or climbing during the nonimmobile time. Thus, although a behavioral consequence could be elicited by the chronic administration of these antidepressants, their chronic treatment failed to affect the secretion of corticosterone or progesterone in response to swim stress.

Discussion

The present studies demonstrated that acute administration of fluoxetine, as well as swim stress, induced a robust increase in corticosterone and progesterone secretion. Because depressed patients can have chronically elevated cortisol levels (Gibbons, 1964; Sachar, 1975), it seems paradoxical that an acute response to an antidepressant (*i.e.*, induction of corticosterone secretion by fluoxetine) is similar to a symptom manifested in some depressed patients. However, the recognized requirement for chronic antidepressant administration to achieve a therapeutic response in depressed patients, which includes a reduction in their hypersteroid secretion, demonstrates that acute pharmacological

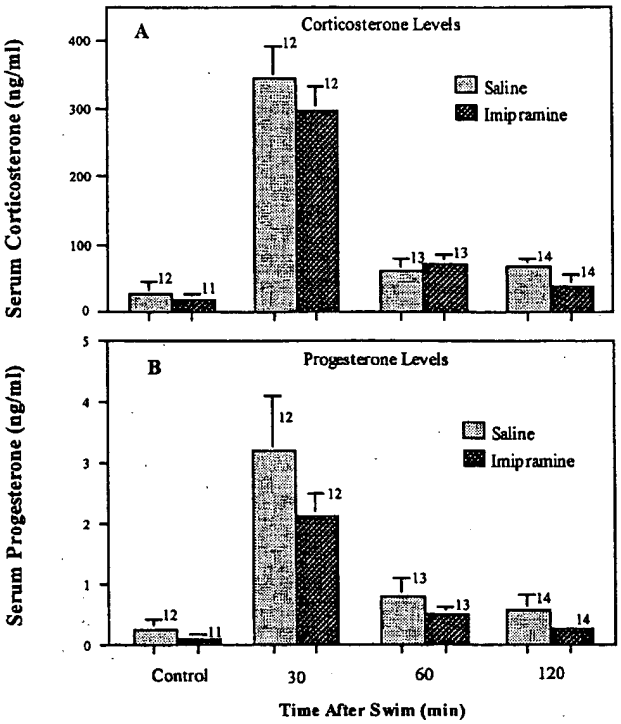

Fig. 7. Lack of effect of chronic imipramine treatment on swim-induced corticosterone or progesterone. Rats were injected daily with imipramine (20 mg/kg) for 21 days before processing in the forced swim test as described under "Materials and Methods." After the test swim (20–24 hr after the final drug or saline injection), the rats were transferred to their home cages and sacrificed by decapitation at the indicated times after the swim for collection of trunk blood. Rats that received chronic injections of saline or imipramine and were not swum were sacrificed 20 to 24 hr after the final injection. The number of rats for each time and treatment are indicated above the bars. Data are expressed as the mean ± S.E.M. for each group.

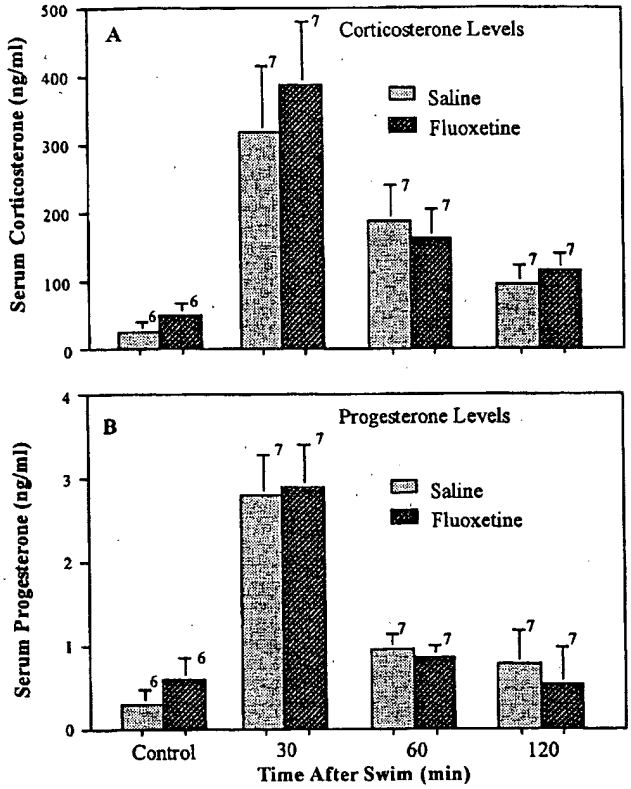

Fig. 8. Lack of effect of chronic fluoxetine treatment on swim-induced corticosterone or progesterone. Rats were injected daily with fluoxetine (5 mg/kg) for 21 days before processing in the forced swim test as described under "Materials and Methods." After the test swim (20–24 hr after the final drug or saline injection), the rats were transferred to their home cages and sacrificed by decapitation at the indicated times after the swim for collection of trunk blood. Rats that received chronic injections of saline or fluoxetine and were not swum were sacrificed 20 to 24 hr after the final injection. The number of rats for each time and treatment are indicated above the bars. Data are expressed as the mean ± S.E.M. for each group.

properties of fluoxetine do not account for the clinical efficacy of this drug class. One hypothesis tested in the present work was that chronic treatment with antidepressants would induce adaptive changes leading to an attenuation of the acute fluoxetine and stress-induced adrenal cortical activation. Our goal was to characterize a model system that could be used to explore antidepressant-induced neurochemical adaptation relevant to neural mechanism(s) by which antidepressants reduce the hypersecretion of cortisol in depressive illness.

Acute effects of fluoxetine and swim stress on corticosterone and progesterone secretion. Other investigators previously have observed that swim stress can increase progesterone as well as corticosterone secretion (Purdy *et al.*, 1991). Although acute fluoxetine has been reported to increase corticosterone secretion (Stark *et al.*, 1985; Fuller and Snoddy, 1990), our data are the first to demonstrate that acute fluoxetine administration also induces secretion of progesterone. Increased progesterone secretion during the menstrual cycle and administration of synthetic progesterone in oral contraceptives have been associated with depressive illness in some women (Backstrom, 1995; Crammer, 1986; Wagner and Berenson, 1994). However, many women do not experience depression while taking progestins in oral contraceptives, and anecdotal evidence suggests that the high levels of progesterone that exist during pregnancy are associated with a state of well-being. Thus, additional work is required to determine the psychophysiological consequences of alterations in progesterone levels in males and females. Little is known about the functional consequences of increased progesterone secretion in males, but plasma-derived progesterone can serve as a precursor in the brain for the synthesis of potent neuroactive steroids which can affect γ-aminobutyric acid-mediated neurotransmission (Majewska *et al.*, 1986; Morrow *et al.*, 1987; Purdy *et al.*, 1991; Paul and Purdy, 1992). Such data indicate that adrenal-derived progesterone in males could be neurochemically relevant.

Possible mechanisms of acute fluoxetine-induced corticosterone and progesterone secretion. Inhibition of the serotonin transporter by fluoxetine results in an increased extracellular fluid level of serotonin (Fuller and Snoddy, 1990). Therefore, the most probable mechanism by which acute fluoxetine induces activation of the brain-pituitary-adrenal axis would be by increasing the synaptic concentration of serotonin, with consequent increased activation of a serotonin receptors. Nonetheless, the specific serotonergic receptor mechanism involved in the neuroendocrine activation by fluoxetine is unknown. Both 5HT-2 and 5HT-1A receptor agonists have been shown to increase corticosterone

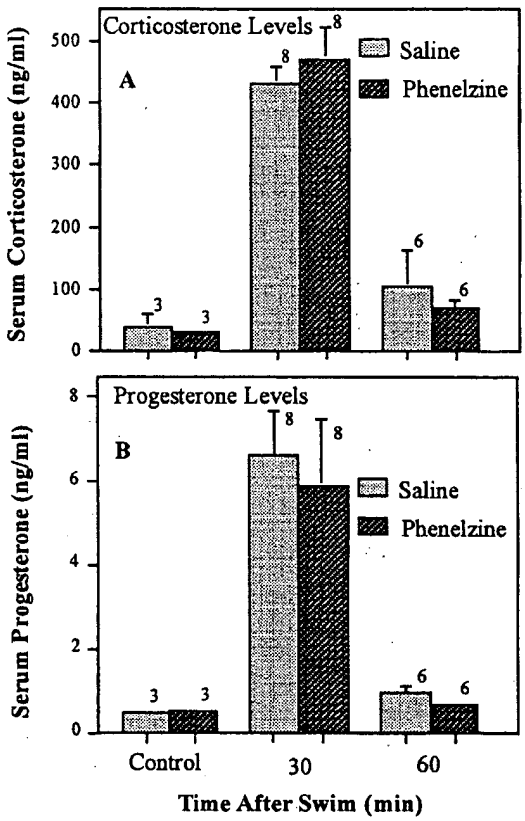

Fig. 9. Lack of effect of chronic phenelzine treatment on swim-induced corticosterone or progesterone. Rats were injected daily with phenelzine (PHEN, 5 mg/kg) for 21 days before processing in the forced swim test as described under "Materials and Methods." After the test swim (20–24 hr after the final drug or saline injection), the rats were transferred to their home cages and sacrificed by decapitation at the indicated times after the swim for collection of trunk blood. Rats that received chronic injections of saline or phenelzine, and were not swum, were killed 20 to 24 hr after the final injection. The number of rats for each time and treatment are indicated above the bars. Data are expressed as the mean ± S.E.M. for each group.

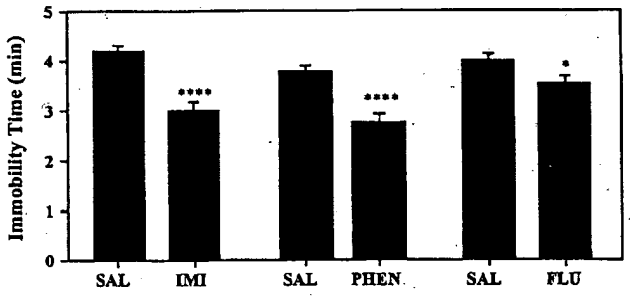

Fig. 10. Immobility time for rats processed in the forced swim test after chronic antidepressant treatment. Rats were injected with saline (SAL), imipramine (IMI, 20 mg/kg) or fluoxetine (FLU, 5 mg/kg) and immobility time scored in the test swim (see "Materials and Methods"). ****$P < .001$, *$P < .05$ compared with saline.

levels (Urban *et al.*, 1986; Fuller *et al.*, 1986; Koenig *et al.*, 1987; Fuller and Snoddy, 1990) and chronic fluoxetine treatment reduces corticosterone secretion induced by the 5HT-1A agonists (Li *et al.*, 1993,1994). However, 5-HT-1A and 5HT-2 receptor antagonists do not attenuate the acute fluoxetine-induced increase in corticosterone levels (Fuller and Snoddy, 1990). Furthermore, interactions between the two receptor subtypes can not explain the effects of fluoxetine on corticosterone secretion, because coadministration of 5HT-1A and 5HT-2 receptor antagonists also did not block this fluoxetine-induced endocrine response (Fuller and Snoddy, 1990). Consequently, if enhancement of synaptic availability of endogenous serotonin by the fluoxetine is responsible for the adrenal cortical activation, a serotonin receptor subtype other than 5HT-1A or 5HT-2 may be involved. Although one potential candidate is the recently cloned 5HT-7 receptor, which is enriched in the hypothalamus (To *et al.*, 1995; Sleight *et al.*, 1995), no direct information currently documents specific 5HT receptor subtypes in the control of fluoxetine-induced steroid release.

Effect of chronic antidepressant administration on fluoxetine-induced steroid secretion. Chronic administration of all antidepressant drug types which inhibit uptake of serotonin, including fluoxetine (Stark *et al.*; 1985), imipramine and amytriptyline (Randrup and Braestrup, 1977; Wood *et al.*, 1986), blocked the increase in corticosterone and progesterone induced by the acute fluoxetine challenge, without affecting base-line levels of these steroids. Likewise, chronic administration of desipramine, which potently inhibits its norepinephrine uptake with minimal effect on serotonin uptake (Randrup and Braestrup, 1977), also antagonized fluoxetine-induced steroid secretion. These findings suggest that chronic inhibition of either serotonin uptake or noradrenergic uptake results in adaptive changes that markedly attenuate the ability of fluoxetine to induce secretion of corticosterone and progesterone. Unlike the effects of chronic treatment with fluoxetine and tricyclic antidepressant drugs, chronic treatment with the MAO inhibitors, phenelzine and tranylcypromine, did not result in a significant attenuation of the corticosterone and progesterone responses to the acute challenge with fluoxetine. Thus, the attenuation of fluoxetine-induced steroid secretion is not a general feature of all antidepressant drugs.

Possible adaptive mechanisms responsible for the antagonism of the steroid release by chronic antidepressant treatments. After chronic fluoxetine treatment, *in vivo* microdialysis studies have shown that extracellular fluid levels of serotonin are markedly elevated after the last chronic fluoxetine treatment (Rutter *et al.*, 1994). The increased extracellular level of serotonin induced by fluoxetine after chronic treatment was even greater than after an acute fluoxetine challenge (Rutter *et al.*, 1994). Thus, a reduced effectiveness of fluoxetine to release serotonin after chronic administration does not appear to be responsible for the observed blockade of the steroid release after acute fluoxetine administration. Additionally, serum levels of fluoxetine after acute challenge were similar in groups treated chronically with saline or with the antidepressants that blocked the acute steroid response to fluoxetine challenge. Therefore, an increased metabolism of fluoxetine can not explain the antagonism of fluoxetine-induced steroid release by chronic treatment of selected antidepressant drugs.

Chronic treatment with antidepressant drugs which block serotonin or norepinephrine uptake sites must result in a substantial neurochemical adaptation in neural circuits which regulate fluoxetine-induced adrenal hormone secretion, as both types of drugs antagonized this response. Although chronic treatment with fluoxetine has been shown to

reduce corticosterone secretion induced by 5HT-1A agonists, (Li *et al.*, 1993), chronic treatment with desipramine, which also reduces the acute fluoxetine-induced increases in steroid secretion, does not modify 5HT-1A agonist-induced corticosterone secretion (Li *et al.*, 1994). These data, considered with the data of Fuller and Snody (1990) discussed above, suggest that the antagonism of fluoxetine-induced adrenal hormone secretion after chronic antidepressant treatment can not be explained by a reduction in the sensitivity of 5HT-1A receptors.

Chronic desipramine treatment, which antagonizes fluoxetine-induced release of steroids, causes down-regulation of *beta* adrenergic receptor number and function (see Baker and Greenshaw, 1989). Although chronic fluoxetine treatment also antagonizes steroid release by acute fluoxetine administration, chronic fluoxetine does not cause a down-regulation of *beta* adrenergic receptors (Johnson, 1991). As observed for the tricyclic antidepressant drugs (Baker and Greenshaw, 1989), chronic administration of MAO inhibitors also induces an adaptive down-regulation of *beta* adrenergic receptors; however, this class of antidepressants does not have an effect on the acute fluoxetine-induced release of steroids. Collectively, these latter data suggest that antidepressant-induced adaptive changes in noradrenergic receptor function can not explain the present finding that selected antidepressant drugs block acute fluoxetine-induced release of steroids.

Chronic administration of fluoxetine, tricyclic antidepressants and MAO inhibitors has been shown to reduce the expression of mRNA for CRF in the paraventricular nucleus of the hypothalamus (Brady *et al.*, 1991, 1992). However, this action is apparently not sufficient to explain the present results, because MAO inhibitors are ineffective in reducing fluoxetine-induced hormonal secretion. Chronic fluoxetine administration also has been shown to down-regulate the 5-HT-7 receptor subtype in hypothalamic membrane preparations after chronic fluoxetine treatment (Sleight *et al.*, 1995); however, it is not known whether chronic desipramine affects this 5-HT receptor subtype. Thus, it can be concluded that the adaptive mechanism(s) by which chronic antidepressant treatment blocks the acute action of fluoxetine to stimulate corticosterone and progesterone secretion has yet to be identified.

Chronic antidepressant treatments on forced swim-induced immobility and corticosterone and progesterone secretion. The final aspect of our investigation was to determine whether chronic antidepressant treatment would antagonize the release of corticosterone and progesterone induced by swim stress (Purdy *et al.*, 1991). This particular stressor was chosen because the protocol of forced swim is used to screen for antidepressant action (Porsolt *et al.*, 1978). As expected from previous work (Duncan *et al.*, 1985, 1996), chronic administration of the antidepressant drugs fluoxetine, imipramine and phenelzine resulted in a significant reduction in the immobility in the forced swim test paradigm. Even though chronic administration of the antidepressants produced a behavioral change induced by forced swim, swim stress-induced secretion of these steroids was not affected by such chronic antidepressant exposure. The inability of chronic treatment with fluoxetine and imipramine to modify adrenal cortical secretion in response to swim stress suggests that a generalized down-regulation of the brain-pituitary-adrenal axis was not induced by the antidepressant drug treatments.

In contrast to the present data showing no effect of chronic antidepressant treatment on swim-induced activation of the brain-pituitary-adrenal axis, Reul *et al.* (1993) found that chronic administration of amytriptyline reduced the corticosterone response induced by placing rats in a novel environment. Differences in the nature of these stressors and the possible activation of differing neural pathways by the stressors could explain the ability of chronic treatment to block the stress response induced by the novel environment but not affect the increase in corticosterone induced by the swim. On the other hand, the magnitude of the stress response could be a consideration, because swim stress could be expected to be more stressful than novelty stress. In this regard, swim stress typically increased corticosterone levels to 300 to 400 ng/ml, whereas in the study of Reul *et al.*(1993), the novelty stress condition increased corticosterone to about 250 ng/ml. Thus, one interpretation could be that chronic antidepressant treatment could not overcome the hormonal response of the more severe swim stress compared with the stress of novel environment. However, contrary to this interpretation, acute fluoxetine treatment increased steroids to a level as great as that observed with swim stress, yet this increase by the acute fluoxetine challenge was blocked completely by the chronic antidepressant treatments.

Therefore, the differential effects observed for the antidepressant treatments on swim stress-induced and fluoxetine-induced alterations in corticosterone and progesterone secretion may involve differing neural mechanisms associated with regulation of adrenal steroid secretion. In this regard, at least two distinct pathways controlling CRF release have been proposed previously, one originating from brainstem structures and the other involving projections from limbic regions of the forebrain (Herman and Cullinan, 1997; Sawchenko, 1990). Whether altered activity in these distinct pathways contributes to the differential effects of chronic antidepressant treatment on fluoxetine- and stress-induced adrenal steroid secretion deserves investigation.

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References

Backstrom T (1995) Symptoms related to the menopause and sex steroid treatments. *Ciba Found Symp* 191:171–180.

Baker GB and Greenshaw AJ (1989) Effects of long-term administration of antidepressants and neuroleptics on receptors in the central nervous system. *Cell Mol Neurobiol* 9:1–44.

Banki CM, Bissette G, Arato M, O'Connor L and Nemeroff CB (1987) CSF corticotropin-releasing factor-like immunoreactivity in depression and schizophrenia. *Am J Psychiatry* 144:873–877.

Brady LS, Whitfield, Jr HJ, Fox RJ, Gold PW and Herkenham, M (1991) Long-term antidepressant administration alters corticotropin-releasing hormone, tyrosine hydroxylase, and mineralocorticoid receptor gene expression in rat brain. *J Clin Invest* 87:831–837.

Brady LS, Gold PW, Herkenham M, Lynn AB and Whitfield HJ Jr (1992) The antidepressants fluoxetine, idazoxan and phenelzine alter corticotropin-releasing hormone and tyrosine hydroxylase mRNA levels in rat brain: therapeutic implications. *Brain Res* 572:117–125.

Breier A and Buchanan RW (1992) The effects of metabolic stress on plasma progesterone in healthy volunteers and schizophrenic patients. *Life Sci* 51:1527–1534.

Carroll BJ, Feinberg M, Greden JF, Tarika J, Albala AA, Haskett RF, James NM, Kronfol Z, Lohr N, Steiner Mde Vigne JP and Young E (1981) A specific laboratory

test for the diagnosis of melancholia: Standardization, validation, and clinical utility. *Arch Gen Psychiatry* 38:15–22.
Crammer JL (1986) Premenstrual depression, cortisol and estradiol treatment. *Psychol Med* 16:451–455.
Deis RP, Leguizamon E and Jahn GA (1989) Feedback regulation by progesterone of stress-induced prolactin release in rats. *J Endocrinol* 120:37–43.
Detke MJ, Rickels M and Lucki I (1995) Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology* 121:66–72.
Duman RS, Heninger GR and Nestler EJ (1994) Adaptations of receptor-coupled signal transduction pathways underlying stress- and drug-induced neural plasticity. *J Nerv Ment Dis* 182:692–700.
Duncan GE, Knapp DJ, Johnson KB and Breese GR (1996) Functional Classification of antidepressants based upon antagonism of swim stress-induced Fos-like immunoreactivity. *J Pharmacol Exp Ther* 277:1076–1089.
Duncan GE, Paul IA, Harden JK, Mueller RA, Stumpf W and Breese GR (1985) Rapid down regulation of β-adrenergic receptors by combining antidepressant drugs with forced swim: A model of antidepressant-induced neural adaptation. *J Pharmacol Exp Ther* 234:402–408.
Ettigi PG and Brown GM (1977) Psychoneuroendocrinology of affective disorders: An overview. *Am J Psychiatry* 134:493–501.
Fuller RW and Snoddy HD (1990) Serotonin receptor subtypes involved in the elevation of serum corticosterone concentration in rats by direct- and indirect-aging serotonin agonists. *Neuroendocrinology* 52:206–211.
Fuller RW, Snoddy HD and Molloy BB (1986) Central serotonin agonist actions of LY 165163, 1-(m-trifluoromethylpheny)-4(p-aminophenylethyl)-piperazine, in rats. *J Pharmacol Exp Ther* 239:454–459.
Ganong WF and Forsham PH (1960) Adenohypophysis and adrenal cortex. *Annu Rev Physiol* 22:579–614.
Gibbons JL (1964) Cortisol secretion rate in depressive illness. *Arch Gen Psychiatry* 10:572–575.
Heninger GR and Charney DS (1987) Mechanisms of action of antidepressant treatments: implications for the etiology and treatment of depressive disorders, in *Psychopharmacology: The Third Generation of Progress* (Meltzer HY, ed) pp 535–544, Raven, New York.
Herman JP and Cullinan WE (1997) Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* 20:78–84.
Holsboer F and Barden N (1996) Antidepressants and hypothalamic-pituitary-adrenocortical regulation. *Endocr Rev* 17:187–205.
Holsboer F, von Bardeleben U, Wiedemann K, Muller OA and Stalla GK (1987) Serial assessment of corticotropin-releasing hormone response after dexamethasone in depression-Implications for pathophysiology of DST non-suppression. *Biol Psychiatry* 22:228–234.
Johnson AM (1991) The comparative pharmacological properties of selective serotonin re-uptake inhibitors in animals, in *Perspectives in Psychiatry*. vol 1: *Selective Serotonin Re-uptake Inhibitors* (Feighner JP and Boyer WF, eds) pp 37–70, John Wiley and Sons, New York.
Katz MM, Koslow SH, Maas J, Frazer A, Secunda S, Bowden CL, Casper RC, Croughan J, Kocsis J and Redmond E (1987) The timing, specificity, and clinical prediction of tricyclic drug effects in depression. *Psychol Med* 17:297–309.
Klein DF and Davis JM (1969) *Diagnosis and Drug Treatment in Psychiatric Disorders*. Williams & Wilkins Co., Baltimore.
Koenig JI, Gudelsky GA and Meltzer HY (1987) Stimulation of corticosterone and β-endorphin secretion in the rat by selective 5-HT receptor subtype activation. *Eur J Pharmacol* 45:305–310.
Li Q, Levy AD, Cabrere TM, Brownfield MS, Battaglia G and Van de Kar LD (1993) Long-term fluoxetine, but not desipramine, inhibits the ACTH and oxytocin responses to the 5-HT1A agonist, 8-OH-DPAT, in male rats. *Brain Res* 630:148–156.
Li Q, Brownfield MS, Levy AD, Battaglia G, Cabrera TM and Van de Kar LD (1994) Attenuation of hormone responses to the 5-HT1A agonist isapirone by long-term treatment with fluoxetine, but not desipramine, in male rats. *Biol Psychiatry* 36:300–308.
Linkowski P, Mendlewicz J, Kerkhofs M, Leclercq R, Goldstein J, Brasseur M, Copinschi G and Van Cauter E (1987) 24-hour profiles of adrenocorticotropin, cortisol, and growth hormone in major depressive illness: Effect of antidepressant treatment. *J Clin Endocrinol Metab* 65:141–152.
Majewska MD, Harrison NL, Schwartz RD, Barker JL and Paul SM (1986) Steroid hormone metabolites and barbiturate-like modulators of the GABA receptor. *Science* 232:1004–1007.
Meikle AW and Tyler FH (1977) Potency and duration of action of glucocorticoids. *Am J Med* 63:200–207.
Morrow AL, Suzdak PD and Paul SM (1987) Steroid hormone metabolites potentate GABA receptor-mediated chloride ion flux with nanomolar potency. *Eur J Pharmacol* 142:483–485.
Nemeroff CB, Widerlow E, Bissette G, Walleus H, Karlsson I, Eklund K, Klits CD, Loosen PT and Vale W (1984) Elevated concentrations of CSF corticotropin-releasing actor-like immunoreactivity in depressed patients. *Science* 226:1342–1344.
Nequin LG, Alvarez JA and Campbell CS (1975) Alterations in steroid and gonadotropin release resulting from surgical stress during the morning of proestrus in 5-day cyclic rats. *Endocrinology* 97:718–724.
Paul SM and Purdy RH (1992) Neuroactive steroids. *FASEB J* 6:2311–2322.
Porsolt RD, Anton A, Blavet N and Jalfre M (1978) Behavioral despair in rats: A new model sensitive to antidepressant treatments. *Eur J Pharmacol* 47:379–391.
Purdy RH, Morrow AL, Moore PH, Jr and Paul SM (1991) Stress-induced elevations of γ-aminobutyric acid type A receptor-active steroids in the rat brain. *Proc Natl Acad Sci USA* 88:4553–4557.
Raadsheer FC, van Heerikhuize JJ, Lucassen PJ, Hoogendijk WJG, Tilders FJH and Swaab DF (1995) Corticotropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression. *Am J Psychiatry* 152:1372–1376.
Randrup A and Braestrup C (1977) Uptake inhibition of biogenic amines by newer antidepressant drugs. Relevance to the dopamine hypothesis of depression. *Psychopharmacology* 52:308–314.
Reul JMHM, Stec I, Sodr M and Holsboer F (1993) Chronic treatment of rats with the antidepressant amytriptyline attenuates the activity of the hypothalamic-pituitary-adrenocortical system. *Endocrinology* 133:312–320.
Rubin RT, Poland RE, Lesser IM, Winston RA and Boldgett ALN (1987) Neuroendocrine aspects of primary endogenous depression. *Arch Gen Psychiatry* 44:238–336.
Rutter JJ, Gundlah C and Auerbach SB (1994) Increase in extracellular serotonin produced by uptake inhibitors is enhanced after chronic fluoxetine treatment. *Neurosci Lett* 171:183–186.
Sachar EJ (1975) Neuroendocrine abnormalities in depressive illness, in *Topics in Psychoendocrinology* (Sachar EJ, ed) pp 256–298, Grune and Stratton, New York.
Sawchenko PE (1990) The final common path: Issues concerning the organization of central mechanisms controlling corticotropin secretion, in *Stress Neurobiology and Neuroendocrinology* (Brown MR, Koob GF and Rivier C, eds) pp 55–71. New York: Marcel Dekker, publisher.
Schaeffer C and Aron CL (1987) Stress-related effects on the secretion of progesterone by the adrenals in castrated male rats presented to stimulus males. Involvement of estrogen. *Acta Endocrinol* 114:440–445.
Schmider J, Lammers, C-H, Gotthardt U, Dettling M, Holsboer F and Heuser IJE (1995) Combined dexamethasone/corticotropin releasing hormone test in acute and remitted manic patients, in acute depression, and in normal controls. *Biol Psychiatry* 38:792–802.
Sleight AJ, Carolo C, Petit N, Zwingelstein C and Bourson A (1995) Identification of 5-hydroxytryptamine$_7$ receptor binding sites in rat hypothalamus: Sensitivity to chronic antidepressant treatment. *Mol Pharmacol* 47:99–103.
Souetre E, Salvati E, Belugou JL, Pringuey D, Candito M, Krebbs B, Ardisson JL and Darcourt G (1989) Circadian rhythms in depression and recovery: Evidence for blunted amplitude as the main chronobiological abnormality. *Psychiatry Res* 28: 263–278.
Stark P, Fuller RW and Wong DT (1985) The pharmacologic profile of fluoxetine. *J Clin Psychiatry* 46:7–13.
Steiger A, Von Bardeleben V, Herth T and Holsboer F (1989) Sleep electroencephalogram and nocturnal secretion of cortisol and growth hormone in male patients with endogenous depression before treatment and after recovery. *J Affect Dis* 16:189–195.
Stokes PE, Pick GR, Stoll PM and Nunn WD (1975) Pituitary-adrenal function in depressed patients: Resistance to dexamethasone suppression. *J Psychiatr Res* 12:271–281.
To ZP, Bonhaus DW, Elgen RM and Jakeman LB (1995) Characterization and distribution of putative 5-ht$_7$ receptors in guinea-pig brain. *Br J Pharmacol* 115:107–116.
Urban JH, Van de Kar LD, Lorens SA and Bethea CL (1986) Effect of the anxiolytic drug buspirone on prolactin and corticosterone secretion in stressed and unstressed rats. *Pharmacol Biochem Behav* 25:457–462.
Wagner KD and Berenson AB (1994) Norplant-associated major depression and panic disorder. *J Clin Psychiatry* 55:478–480.
Wood MD, Broadhurst AM and Wylie MG (1986) Examination of the relationship between the uptake system for 5-hydroxytryptamine and the high affinity ^{3}H-imipramine binding site. *Neuropharmacology* 25:519–525.

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